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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Our previous studies demonstrate that BRCA1 regulates *GADD45*, a p53-regulated and stress-inducible gene that plays an important role in cellular response to DNA damage. Currently, we investigated the biochemical mechanism(s) involved in the *GADD45*-activated G2-M arrest and found that expression of Gadd45 protein via the tet-off inducible system resulted in cell cycle G2/M arrest. Gadd45-induced G2-M arrest was shown to require normal cellular p53 function, but is independent of p38 kinase activity. Interestingly, induction of Gadd45 protein resulted in a reduction of nuclear cyclin B1 protein, whose nuclear localization is critical for the completion of G2-M transition. The reduced nuclear cyclin B1 levels correlated with inhibition of Cdc2/cyclin B1 kinase activity. In addition, we demonstrated that BRCA1 activation of the *GADD45* promoter was mediated through the OCT-1 and CAAT motifs located at the *GADD45* promoter region. Site-directed mutations of both OCT-1 and CAAT motifs abrogated induction of the *GADD45* promoter by BRCA1. Further more, BRCA1 was found associated with the Gadd45 promoter, probably through physical interaction with Oct-1 and NF-YA, which directly bind to the Gadd45 promoter. The current study demonstrates a novel pathway (BRCA1-GADD45) involved in cellular response to DNA damage.

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1. INTRODUCTION

Mutations of the breast cancer susceptibility gene, BRCA1, are associated with more than half the cases of hereditary breast cancer (1-3). It has been demonstrated that BRCA1 play an important role in genotoxic stress-induced cell cycle checkpoint, apoptosis and DNA repair. Those cellular responses are thought to maintain genomic fidelity. Deregulation of genomic stability is closely associated with malignant transformation and tumorigenesis (4-10). However, the molecular mechanism by which BRCA1 plays a role in maintaining genomic integrity remains to be further defined. The current project is to characterize the molecular pathway, which mediates BRCA1 ' s role in the control of cell cycle G2-M checkpoint in response to DNA damage. The proposed studies will provide the understanding of how BRCA1 participates in maintenance of genomic stability and provide the insight to development of novel anticancer drugs. Our previous and current work indicates that BRCA1 transcriptionally activates Gadd45, a p53-regulated and DNA damage-inducible gene that may play an important role in cell cycle G2-M checkpoints, apoptosis and DNA repair in response to DNA damage (11-17). We speculated that the role of BRCA1 in cell cycle G2-M checkpoint is mediated through Gadd45 and *GADD45* is a targeted gene of BRCA1. Therefore, two major tasks have been proposed in this study. (1). To define the role of Gadd45 in cell cycle G2-M arrest and the possible underlying mechanism. (2). To determine the biochemical and molecular mechanism by which BRCA1 regulates Gadd45.

2. BODY

In the second year of this grant, we have achieved significant research progression in both proposed tasks. These are summarized as the following individually.

Task 1: To define the role of Gadd45 in cell cycle G2-M arrest and the possible underlying mechanism.

1. **To further define the role of Gadd45 in cell cycle G2-M growth arrest in response to DNA damage.** In the first year of the grant, we have successfully established two Gadd45-inducible cell lines via the tet-off system in Hela (p53 negative status) and HCT116 (with wt p53) cells. In these cell lines, expression of Gadd45 protein is precisely controlled through the withdrawal of tetracycline. Therefore, Gadd45 protein is highly induced in the absence of tetracycline (**see appendix-page 40**).

Using these Gadd45-inducible cell lines, we have demonstrated (1). Induction of Gadd45 protein significantly suppresses cell growth in a typical growth survival assay (**page 40**). (2) Induction of Gadd45 protein in HCT116 cell line (wt p53) results in cell arrest in cell cycle G2-M phase, but induction of Gadd45 protein in Hela cells (deleted p53) fail to generate G2-M accumulation, suggesting that the Gadd45-activated cell cycle G2-M arrest is dependent on normal cellular p53 function (**page 41**). (3). Gadd45-induced cell cycle G2-M arrest is independent of p38 kinase activity, which is required for the initiation of G2-M checkpoint (**page 42**). (4). Inducible expression of Gadd45 protein is shown to alter the subcellular distribution of cyclin B1 and results in reduction of nuclear cyclin B1 levels (**page 43**). Taken together, these results

demonstrate that Gadd45, as a BRCA1-targeted effector, is capable of mediating BRCA1-induced cell cycle arrest (**results are shown in the manuscript accepted by Oncogene, see appendix**).

2. **BRCA1 plays a role in cell cycle G2-M arrest**. We have developed several isogenic cell lines, where BRCA1 expression is inhibited by expression of antisense BRCA1 mRNA. In the western analysis, the expression of endogenous BRCA1 protein levels in these isogenic lines is about one fifth of parental cells. The preliminary data demonstrated that the UV-induced cell cycle checkpoint in BRCA1-antisense lines (BRCA1-deficient cells) was impaired, suggesting that BRCA1 is an important player in cell cycle G2-M checkpoint. In the near future, detailed analysis of cell cycle G2-M arrest will be further conducted, including, evaluation of mitotic index and cyclinB1/Cdc2 kinase activity.

3. **The capability of BRCA1-inducing G2-M arrest is impaired in Gadd45-deficient cells.** We have introduced BRCA1 expression vector into Gadd45 deficient cells (ras-immortalized embryonic mouse fibroblast derived from gadd45 knockout mice) and examined the cell cycle profile.

However, we are experiencing the problem in establishing BRCA1-inducible cell lines. Although the Northern analysis demonstrated that BRCA1 mRNA was induced, we failed to detect appreciable induction of BRCA1 protein. We are trying to solve this issue in a short period of time.

4. **BRCA1 growth suppressive-properties are impaired in Gadd45-deficient cells**. We have introduced the BRCA1 expression vector into Gadd45 deficient cells (ras-immortalized embryonic mouse fibroblast derived from gadd45 knockout mice) and examined the growth suppression. We found that BRCA1-induced growth suppression is partially abrogated in Gadd45-deficient cells, indicating that Gadd45 plays a role in mediating growth inhibition by BRCA1.

Task 2: To determine the biochemical and molecular mechanism by which BRCA1 regulates Gadd45.

1. In the first year of the grant, several major findings have been obtained. (1). Expression of BRCA1 upregulates Gadd45 mRNA in multiple human cell lines. (2). BRCA1 can activate the Gadd45 promoter in a p53-independent manner. (3). The BRCA1-responsive elements are localized at the region of the Gadd45 promoter between –107 to –57. Deletion of this region has been shown to abrogate BRCA1 activation of the Gadd45 promoter (**Results were presented in the 2001 annual report**).

Based on these findings, a series of experiments were performed to explore the biochemical mechanism. We have obtained the following results:

(1). According to the analysis of the promoter sequence, we found two Oct-1 sites and one CAAT box in the BRCA1-responsive region. Deletion of this region disrupted the BRCA1 activation of the Gadd45 promoter (**results are shown in JBC paper in appendix, page 12, partial results were reported in the 2001 annual report**).

(2). We made mutations in Oct-1 and CAAT motifs of the Gadd45 promoter, and found that mutations made in all Oct-1 and CAAT sites completely abolished BRCA1 activation of the Gadd45 promoter (**results are shown in JBC paper in the appendix, page 13**).

(3). Using a "pull-down" assay, we have shown that BRCA1 protein binds to the Gadd45 promoter. This binding is mediated through both Oct-1 and NF-YA (CAAT box binding protein) transcription factors because depletion of these proteins results in abrogation of BRCA1 protein binding to the Gadd45 promoter DNA (**results are shown in JBC paper in the appendix, page 14**).

(4). In immunoprecipitation assays, the BRCA1 protein has been shown to physically interact with Oct-1 and NF-YA proteins (**results are shown in JBC paper in the appendix, page 14**).

3. KEY RESEARCH ACCOMPLISHMENTS

The major accomplishments in this project during the past year are summarized: (1). We have further demonstrated Gadd45, as a BRCA1-targeted gene, plays an important role in the cell cycle G2-M checkpoint. The underlying mechanism is that Gadd45 protein associates with Cdc2 kinase, leading to dissociation of the Cdc2/Cyclin B1 complex. The "free" Cyclin B1 will then be pumped out of the nucleus to the cytoplasmic compartment for degradation. This data provides a precise explanation for the machinery involved in the BRCA1/Gadd45 pathway induced cell cycle growth arrest in response to DNA damage (**appendix, Oncogene paper in press**). (2). We have demonstrated that BRCA1 activates the Gadd45 promoter through Oct-1 and NF-YA transcription factors. These findings indicate that BRCA1 can upregulate its targeted genes through protein-protein interactions and provide a novel mechanism by which BRCA1 participates in transcriptional regulation. This finding will significantly broaden the role of BRCA1 in regulation of its targeted genes (**appendix, JBC paper**).

4. REPORTABLE OUTCOMES

(1). One paper has been published in the Journal of Biological Chemistry and one paper is in press in Oncogene. Those are decent journals in the cancer research and biology fields (**Appendix**).

(2). Based on results produced in this grant, we have successfully obtained one NIH R01 grant in early 2002 (**appendix, page 47**).

(3). One abstract has been presented in the 93rd Annual Meeting of the American Association of Cancer Research in San Francisco, CA, 2002 (**appendix, page 46**).

(4). Under the support from the DOD, one postdoctoral fellow has been hired to obtain training in my laboratory.

5. CONCLUSION

Overall, this DOD-funded project has been going very well during the second year. The findings from this project have demonstrated a novel pathway (BRCA1-Gadd45) in cellular response to DNA damage, indicating that the role of BRCA1 in the maintenance of genomic stability may be mediated through Gadd45, a DNA damage-inducible gene that plays a role in the cell cycle checkpoint. Future work will be focused on defining the detailed biochemical and molecular mechanism by which the pathway plays an important role in preventing breast cancer. Completion of this project will provide insight into the development of new anti-breast cancer drugs.

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Appendices

1. **Reprint published in JBC (Fan et al, 2002)**
2. **Manuscript in press in Oncogene (Jin at al, 2002)**
3. **Abstract presented in 2002 annual AACR meeting**
4. **NIH R01 grant award notice**

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BRCA1 Regulates *GADD45* through Its Interactions with the OCT-1 and CAAT Motifs*

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***BRCA1*, a breast and ovarian cancer susceptibility gene, has been implicated in gene regulation. Previous studies demonstrate that BRCA1 induces *GADD45*, a p53-regulated and stress-inducible gene that plays an important role in cellular response to DNA damage. However, the mechanism(s) by which BRCA1 regulates *GADD45* remains unclear. In this report, we have shown that BRCA1 activation of the *GADD45* promoter is mediated through the OCT-1 and CAAT motifs located at the *GADD45* promoter region. Site-directed mutations of both OCT-1 and CAAT motifs abrogate induction of the *GADD45* promoter by BRCA1. Both OCT-1 and CAAT motifs are able to confer BRCA1 inducibility in a nonrelated minimal promoter. Physical associations of BRCA1 protein with transcription factors Oct-1 and NF-YA, which directly bind to the OCT-1 and CAAT motifs, are established by biotin-streptavidin pull-down and co-immunoprecipitation assays. Such protein interactions are required for interaction of BRCA1 with the *GADD45* promoter because either immunodepletion of Oct-1 and NF-YA proteins or mutations in the OCT-1 and CAAT motifs disrupt BRCA1 binding to the *GADD45* promoter. These findings indicate that BRCA1 can up-regulate its targeted genes through protein-protein interactions and provide a novel mechanism by which BRCA1 participates in transcriptional regulation.**

Mutations of the breast cancer susceptibility gene, *BRCA1*, are associated with more than half the cases of hereditary breast cancer (1–3). The human *BRCA1* gene encodes a nuclear protein of 1863 amino acids and is expressed in a variety of human tissues (3, 4). Neoplastic development in *BRCA1* mutation carriers is generally accompanied by loss of the wild-type allele, suggesting BRCA1 protein may function as a tumor suppressor. A number of observations have implicated *BRCA1* in cellular response to DNA damage. BRCA1 associates and colocalizes with Rad51 protein and may be involved in DNA recombination. Following DNA damage, BRCA1 becomes hyperphosphorylated by ATM (5) and hCds1/Chk2 (6) and relocalizes to complexes containing proliferating cell nuclear antigen (7). Additionally, *BRCA1* plays an important role in the transcription-coupled repair (8) and in the control of cell cycle arrest following DNA damage (9, 10). Recently, multiple reports (11–13) have suggested that *BRCA1* might also play a role in apoptosis. Therefore, through its functions in DNA repair process, apoptosis, and cell cycle arrest, *BRCA1* plays an important role in the maintenance of genomic integrity. This is strongly supported by the demonstration that murine embryos carrying a *BRCA1* null mutation exhibit hypersensitivity to DNA damage and chromosomal abnormalities, probably due to defective G_2/M checkpoint control and improper centrosome duplication (14).

GADD45 is a DNA damage-responsive gene and is induced by a wide spectrum of genotoxic stress agents, including ionizing radiation, UV radiation, methyl methanesulfonate (MMS),[1] and medium starvation (15–17). It has been shown that induction of *GADD45* after DNA damage is mediated via both p53-dependent (18, 19) and -independent pathways (20). Expression of Gadd45 protein suppresses cell growth (21, 22). Gadd45 protein is able to associate with multiple important cellular proteins, including proliferating cell nuclear antigen (23), p21 (24, 25), Cdc2 (26), core histone (27), and MTK1/MEKK4 (28). Recent findings suggest that *GADD45* is involved in the control of cell cycle checkpoint (29) and apoptosis (28, 30). This argument is further supported by the finding that *GADD45*-null mice exhibit significant genomic instability, which is exemplified by aneuploidy, chromosomal aberrations, and gene amplification, and increased carcinogenesis following treatment with DNA damage (31). Therefore, *GADD45* appears to be an important player in maintenance of genomic stability.

Several lines of evidence support a role for *BRCA1* in transcriptional regulation. BRCA1 has an N-terminal ring finger domain and a C-terminal transcription activation domain that activates transcription when fused to a DNA-binding domain (32). It has been shown that BRCA1 interacts with transcriptional regulators, including p53 (33, 34), c-Myc (35), STAT1 (36), and estrogen receptor (37), and proteins involved in chromatin remodeling including p300/CBP (38) and RBAP46/48-HDAC (39). Expression of BRCA1 activates or suppresses expression of several important cellular proteins, such as $p21^{waf1/CIP1}$ (10) and cyclin B1 (40). Most recently, studies from our group and others (30, 40, 41) have demonstrated that BRCA1 strongly activates *GADD45* in a p53-independent manner. Activation of the *GADD45* promoter requires normal transcription-activating function of BRCA1 because the tumor-derived BRCA1 mutants (1749R and Y1853insA), which lack transcription activity, are unable to activate the *GADD45* pro-

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[1] The abbreviations used are: MMS, methyl methanesulfonate; CAT, chloramphenicol acetyltransferase; PBS, phosphate-buffered saline; GFP, green fluorescence protein; wt, wild type; mut, mutant.

moter (41). However, the molecular mechanism by which BRCA1 up-regulates *GADD45* is complex and may involve the regulatory elements located at either the third intron or the promoter region of *GADD45*. BRCA1 also represses *GADD45* expression through its interaction with ZBRK1 transcription factor (42). Despite the discrepancy of the effect of BRCA1 on *GADD45* transcription, it has been well accepted that *GADD45* is one of the BRCA1 downstream effectors and probably mediates the role of BRCA1 in maintenance of genomic stability.

The transcription factor Oct-1, a member of the POU homeodomain family, is ubiquitously expressed and binds to the AGTCAAAA consensus sequence through its DNA-binding POU domain (43). High affinity Oct-1-binding sites are found in a number of cellular promoters (44), and binding of Oct-1 factor to its consensus motif normally activates Oct-1-regulated genes (45–49). NF-Y is also a ubiquitous transcription factor consisted of three subunits, A–C. NF-Y specifically binds CAAT box motifs, which are found in 30% of eukaryotic promoters (50, 51). Recently, both Oct-1 and NF-YA, but not NF-YB and NF-YC, were found to be induced following treatment with genotoxic agents, indicating that these two transcription factors may participate in cellular response to DNA damage (52, 53).

In this article, we identify OCT-1 and CAAT as the BRCA1-regulatory elements required for BRCA1 activation of the *GADD45* promoter. Disruptions of the OCT-1 and CAAT motifs abolish activation of the *GADD45* promoter by BRCA1. Moreover, BRCA1 physically associates with Oct-1 and NF-YA transcription factors. These results characterize an important molecular mechanism by which BRCA1 regulates *GADD45*.

EXPERIMENTAL PROCEDURES

Plasmid Clones—The following *GADD45* promoter reporter constructs were used: pHG45-CAT1, pHG45-CAT2, pHG45-CAT5, pHG45-CAT7, pHG45-CAT11, pHG-CAT12, and pHG45-CAT13 (53, 54). *GADD45* promoter reporters that contain mutations in either Oct-1 or CATT box motifs (pHg45-CAT11 m1, pHg45-CAT11 m2, pHg45-CAT11 m3, pHg45-CAT11 m4, pHg45-CAT11 m5, pHg45-CAT11 m6, and pHg45-CAT11 m7) were constructed by PCR cloning as described previously (53). pCR3-BRCA1, a construct expressing wt human BRCA1 protein, was provided by B. Weber (see Ref. 10). pC53-SN3, which expresses wild-type p53 protein, was provided by B. Vogelstein (see Ref. 55). PG-CAT−107/−57 was constructed by inserting the *Hin*dIII-*Pst*I DNA fragment corresponding to −107 and −57 of the *GADD45* promoter upstream of a minimal polyomavirus early promoter linked to a CAT gene, which was derived from PG-13 CAT that was provided by Dr. B. Vogelstein. Similarly, PG-OCT-1wt or PG-OCT-1mut was constructed by cloning 5 direct repeats of the intact OCT-1 motif (TGATTTGCATAGCCCTGTGG) or mutated OCT-1 motif (TGGCCTGCATAGCCCTGTGG) upstream of a minimal polyomavirus early promoter linked to a CAT gene via *Hin*dIII- and *Pst*I-cloning sites. In the case of PG-CAATwt or PG-CAATmut, 3 repeats of the intact CAAT motif (TTAACCAATCAC) or mutated CAAT box (TTAACGTATCAC) were cloned into the same reporter plasmids described above.

Cell Culture and Treatment—The human breast carcinoma MCF-7 line, the human lung carcinoma line H1299, and the human colorectal carcinoma line HCT116 were grown in F-12 medium supplemented with 10% fetal bovine serum as described previously (18, 19). For MMS treatment, cells were exposed to medium containing MMS (Aldrich) at 100 μg/ml for 4 h, and then the medium was replaced with fresh medium. For UV radiation, cells in 100-mm dishes were rinsed with PBS and irradiated to a dose of 10 Jm^{-2}. Cells treated with MMS and UV were collected 16 h posttreatment for the CAT assay (20, 54).

Transfection and CAT Assay—4 μg of the *GADD45* promoter reporter constructs and 4 μg of indicated expression vectors were cotransfected into human cells by calcium phosphate precipitation. 40 h later, cells were collected for the CAT assay. In addition, 4 μg of pCMV-GFP plasmid (which expresses green fluorescence protein) was included in each experiment. After transfection, expression of GFP protein was detected by Western blotting assay to determine transfection efficiency. Measurement of CAT activity was carried out as described previously (56). Cells were collected, resuspended in 0.25 M Tris (pH 7.8), and disrupted by three freeze-thaw cycles. Equal amounts of protein were used for each CAT assay. The CAT reaction mixture was incubated at 37 °C overnight, and the CAT activity was determined by measuring the acetylation of ^{14}C-labeled chloramphenicol using thin layer chromatography. Radioactivity was measured directed with Betascope analyzer. The specific CAT activity was calculated by determining the fraction of chloramphenicol that had been acetylated. The relative CAT activity was determined by normalizing the activity of the treated samples to that of the untreated sample. Each value represented the average of at least three separate determinations (54, 56).

Antibodies, Preparation of Nuclear Protein, Immunoprecipitation, and Immunoblotting Analysis—Antibodies against BRCA1, Oct-1, NF-YA, and Jun-D were commercially provided by Santa Cruz Biotechnology (Santa Cruz, CA). For preparation of nuclear protein, exponentially growing HCT116 cells were collected, rinsed with PBS, and resuspended in 200 μl of cold buffer A (10 mM Hepes (pH 7.9); 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM dithiothreitol; 0.5 mM phenylmethylsulfonyl fluoride). Following vortexing, the samples were incubated on ice for 10 min, and Nonidet P-40 was added to a final concentration of 0.5%. After centrifugation, insoluble pellets were resuspended in 100 μl of ice-cold buffer C (20 mM Hepes (pH 7.9); 400 mM KCl; 1 mM EDTA; 1 mM EGTA; 1 mM dithiothreitol; 1 mM phenylmethylsulfonyl fluoride). The samples were placed on ice and subjected to vortexing for 15 s every 10 min, over a period of 40 min. Finally, the samples were centrifuged at 14,000 × *g* for 10 min, and the supernatant (nuclear extract) was collected for further analysis. For immunoprecipitation and immunoblotting analysis, 300 μg of nuclear protein was immunoprecipitated with anti-BRCA1, Oct-1, NF-YA, or Jun-D antibodies and protein A-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h at 4 °C. The immunoprecipitated protein complexes were washed three times with lysis buffer and loaded onto a SDS-PAGE gel. After electrophoresis, the proteins were transferred to Protran membranes. Membranes were blocked in 5% milk, washed with PBST (PBS with 0.1% Tween), and incubated with anti-Oct-1, NF-YA, and BRCA1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Following washing and incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody at 1:4000 in 5% milk, the membranes were washed, and bound horseradish peroxidase was detected by ECL (Amersham Biosciences) and exposure to x-ray film.

Biotin-Streptavidin Pull-down Assay—Four oligonucleotides containing biotin on the 5′-nucleotide of the sense strand were used in the pull-down assays. The sequences of these oligonucleotides are as follows: 1) wt oligo, 5′-GCAGGCTGATTTGCATAGCCCAATGGCCAAGCTGCATGCAAATGAGGCGGA, which corresponds to positions −107 to −57 of the human *GADD45* promoter; 2) mut oligo1, 5′-GCAGGCTGATTTGCATAGCCtgATGGCCAAGCTGCATGCAAATGAGGCGGA, which corresponds to positions −107 to −57 of the human *GADD45* promoter with the CAAT box mutated; 3) mut oligo2, 5′-GCAGGCTGgccTGCATAGCCCAATGGCCAAGCTGCATGCAggcGAGGCGGA, which corresponds to positions −107 to −57 of the human *GADD45* promoter with two OCT-1 motifs mutated; and 4) mut oligo3, 5′-GCAGGCTGATTTGCATAGCCtgATGGCCAAGCTGCATGCA ggcGAGGCGGA, which corresponds to positions −107 to −57 of the human *GADD45* promoter with two OCT-1 sites and one CAAT box mutated.

These oligonucleotides were annealed to their respective complementary oligonucleotides, and 51-bp double-stranded oligonucleotides were gel-purified and used. Nuclear protein was extracted as described earlier. One microgram of each double-stranded oligonucleotide was incubated with 300 μg of nuclear protein for 20 min at room temperature in binding buffer containing 12% glycerol, 12 mM Hepes (pH 7.9), 4 mM Tris (pH 7.9), 150 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 10 μg of poly(dI-dC) competitor. Following the incubation, 30 μl of streptavidin-agarose (Sigma) was added to the reaction and incubated at 4 °C for 4 h. Prior to this step, 300 μl of the original streptavidin-agarose bead preparation was preabsorbed with 500 μl of bovine serum albumin, 50 μg of poly(dI-dC), and 50 μg of sheared salmon sperm DNA for 30 min at 25 °C. The streptavidin-agarose beads were washed three times and resuspended in 300 μl of the binding buffer. The protein-DNA-streptavidin-agarose complex was washed three times with binding buffer and loaded onto a SDS gel. Detection of BRCA1, Oct-1, and NF-YA proteins was performed as described above (54).

RESULTS

Mapping of the BRCA1 Regulatory Elements in the GADD45 Promoter—Our group recently demonstrated (41) that BRCA1 induces expression of *GADD45* mRNA and activates the *GADD45* promoter. As shown in Fig. 1*A*, when pHG45-CAT2, a *GADD45* promoter reporter construct that spans −909 to +144

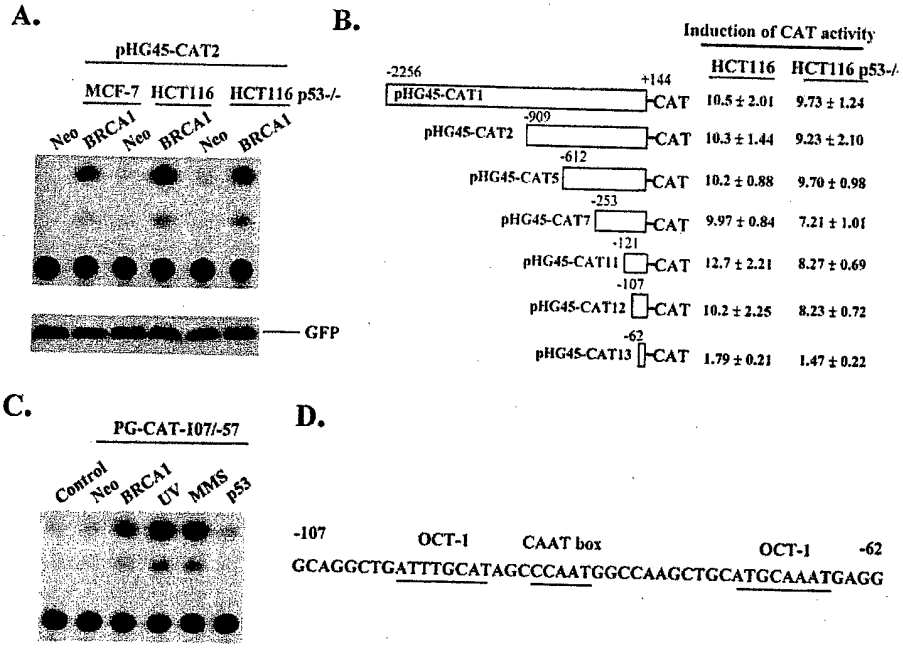

FIG. 1. **Mapping of the BRCA1-regulatory elements in the *GADD45* promoter.** *A*, 4 μg of the *GADD45* promoter CAT reporter constructs pHG45-CAT2 was cotransfected with 4 μg of either pCR3.BRCA1 (BRCA1) or pCMV.neo (Neo) expression vectors into MCF-7, HCT116, or HCT116p53−/− cells using calcium phosphate precipitation. 48 h later, cells were collected, and CAT activity was assayed (see "Experimental Procedures"). To determine transfection efficiency, 4 μg of GFP expression vector was cotransfected with each tested plasmid, and the expression of GFP protein was detected as the internal control of transfection. *B*, 4 μg of the CAT reporter constructs containing the indicated regions of the *GADD45* promoter were cotransfected with pCR3.BRCA1 into HCT116 and HCT116 p53−/− cell lines. CAT assay was carried out as in *A*. *C*, 4 μg of PG-CAT-107/−57 plasmid, where the DNA fragment corresponding to −107 to −57 of the *GADD45* promoter was cloned upstream of a minimal polyomavirus promoter linked to a CAT gene, was cotransfected with either pCR3.BRCA1 (BRCA1), pCMV.neo (Neo), or pC53-SN3 (p53). The CAT assays were performed, and the CAT activities were measured as described under "Experimental Procedures." In some cases, the cells transfected with PG-CAT−107/−57 alone were treated with UV radiation or MMS and followed by CAT assay. All experiments presented in *A* and *C* were repeated at least three times, but only a representative experiment of CAT assay is shown here. *D*, DNA sequence analysis indicates that there are two OCT-1 sites and one CAAT box located at the region of the *GADD45* promoter from −107 to −62.

of the *GADD45* promoter region, was cotransfected with either pCMV.neo (Neo) or pCR3.BRCA1 (BRCA1) into the human breast carcinoma MCF-7 cell line (wt p53), human colorectal carcinoma HCT116 cell line (wt p53), or HCT116 p53−/− cell line (where p53 alleles were deleted via homologue recombination), the *GADD45* promoter reporter was strongly activated in all cell lines regardless of p53 status. To determine transfection efficiency, GFP expression vector was cotransfected with each tested plasmid. The expression of GFP protein detected by immunoblotting analysis indicated that transfection efficiency was similar among different samples with variations less than 20%. To map the BRCA1-responsive elements in the *GADD45* promoter, a series of the *GADD45* CAT reporters that spanned the different regions of the human *GADD45* promoter were constructed. Following cotransfection of these *GADD45* promoter reporter plasmids with the BRCA1 expression vector into human colorectal carcinoma HCT116 and HCT116 p53−/− cells, CAT assays were conducted, and the CAT activities were analyzed. As illustrated in Fig. 1*B*, most of the *GADD45* CAT reporters were strongly activated following expression of BRCA1 protein. With progressive 5′-deletion, pHG45-CAT13 that extended 5′ only to −62 relative to the transcription start site exhibited little induction following expression of BRCA1. These observations indicate that the region between −107 and −62 contains the regulatory elements required for the responsiveness of the *GADD45* promoter to BRCA1 expression.

To confirm if the region from −107 to −62 is responsible for activation of the *GADD45* promoter by BRCA1, we constructed a reporter plasmid designated as PG-CAT−107/−57, where a DNA fragment corresponding to the *GADD45* promoter region between −107 and −57 was cloned upstream of a minimal polyomavirus promoter linked to a CAT reporter gene. This minimal polyomavirus promoter itself is unable to respond to BRCA1 expression or DNA-damaging agents (data not shown). When cotransfected with pCR3.BRCA1 (BRCA1) into HCT116 cells, PG-CAT−107/−57 exhibited induction (Fig. 1*C*). In contrast, both pCMV.neo (Neo) and pC53-SN3 (p53) had no effect on this reporter, indicating that the region between −107 and −57 is capable of conferring the BRCA1 inducibility to a nonrelated promoter reporter. Interestingly, PG-CAT−107/−57 was also shown to be strongly induced by UV radiation and MMS, suggesting that activation of the *GADD45* promoter by BRCA1 and DNA damage might share some common regulatory elements. Inspection of DNA sequence exhibits two OCT-1 motifs and one CAAT box located at this region of the human *GADD45* promoter (Fig. 1*D*).

BRCA1 Activation of the GADD45 Promoter Is Mediated through Both OCT-1 and CAAT Motifs—To determine whether the OCT-1 and CAAT box motifs play roles in activating the *GADD45* promoter following expression of BRCA1, we mutated the OCT-1 or CAAT motifs in *GADD45* promoter CAT reporter constructs (53). It should be noted here that our previous work (54) has demonstrated that there are certain regulatory elements located more upstream of the *GADD45* promoter, such as EGR1/WT1. Therefore, to exclude the influence of such responsive elements, we choose pHG45-CAT11, which only contains the region from −121 to +144 of the *GADD45* promoter. Following cotransfection of these mutants of the *GADD45* promoter reporters into both HCT116 (wt p53) and H1299 cells, where the p53 gene is deleted, induction of CAT activity was determined. As shown in Fig. 2, pHG45-CAT11 exhibited the strongest activation by BRCA1. Single mutation in either OCT-1 or CAAT1 motifs (pHG45-CAT11 m1, pHG45-CAT11 m2, and pHG45-CAT11 m3) had little effect on BRCA1-induced

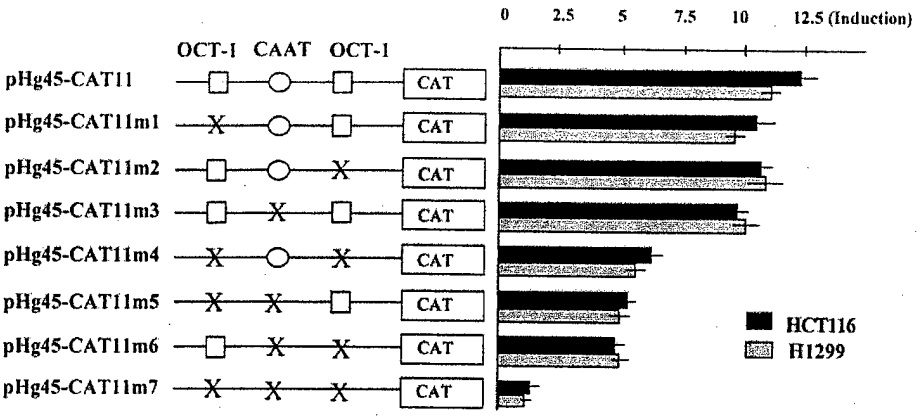

FIG. 2. **Mutations of OCT-1 and CAAT motifs abrogate the activation of the *GADD45* promoter following expression of BRCA1.** 4 μg of the *GADD45* promoter reporter constructs containing the indicated mutations either in OCT-1 sites or in CAAT box were cotransfected with pCR3.BRCA1 into either human colorectal carcinoma HCT116 cells (wt p53) or human lung carcinoma H1299 cells, which contain deleted p53 gene. 40 h later, cells were collected for CAT assay as described under "Experimental Procedures." The values represent the relative induction of the *GADD45* promoter CAT reporters by BRCA1 to that of the Neo-cotransfected controls.

activation of the *GADD45* promoter. However, double mutations in OCT-1 and CAAT sites (pHG45-CAT11 m4, pHG45-CAT11 m5, and pHG45-CAT11 m6) inhibited activation of the *GADD45* promoter by BRCA1, reducing induction of these reporters by 60%. When all three sites were mutated (pHG45-CAT11 m7), the *GADD45* promoter reporter did not exhibit any activation following expression of BRCA1. The responsiveness of the pHG45-CAT11 m7 to BRCA1 expression was observed to be similar to that seen in pHG45-CAT13 (Fig. 1*B*), which only contains the *GADD45* promoter region from −62 to +144. In addition to HCT116 and H1299, we have also examined the activity of the *GADD45* promoter reporters in MCF-7 (wt 53) and HCT116 p53−/− and obtained similar results (data not shown), suggesting that the OCT-1 and CAAT-mediated BRCA1 activation of the *GADD45* promoter does not require p53. These results indicate that both the OCT-1 and CAAT motifs play an important role in BRCA1 activation of the *GADD45* promoter in a p53-independent manner.

We also made mutations in all OCT1 and CAAT motifs in pHG45-CAT2, which covers a longer promoter region between −909 and +144 and determined the BRCA1 activation on this construct. BRCA1 activation of this mutated promoter (pHG45-CAT2ma) was reduced by 70% compared with the pHG45-CAT2 that contains the intact *GADD45* promoter (results not shown). In contrast, BRCA1 activation of the pHG45-CAT11 m7 was completely abolished (Fig. 2). This result is in agreement with our previous finding (54) that there are certain regulatory elements (such as EGR1/WT1) at the upstream region of the *GADD45* promoter. These upstream-responsive elements might also play a role in activation of the *GADD45* promoter by BRCA1, even when mutations were made in OCT1 and CAAT1 motifs.

To determine further the roles of the OCT-1 and CAAT1 motifs in the BRCA1-mediated transcriptional activation, we constructed both OCT-1 and CAAT reporter plasmids, where the multiple repeats of either OCT-1 or CAAT motifs were placed upstream of a polyomavirus minimal promoter that is linked to a chloramphenicol acetyltransferase (CAT) gene. In Fig. 3*A*, PG-OCT-1wt that contains 5 repeats of the intact OCT-1 motifs was transfected with expression vectors for BRCA1, Neo, and Oct-1 into HCT116 cells. PG-OCT-1wt was activated following expression of BRCA1. As an OCT-1 reporter, this construct was also strongly induced by Oct-1 expression. Interestingly, the OCT-1 reporter was responsive to MMS treatment. In contrast, the PG-OCT-1mut that contains 5 repeats of the mutated OCT-1 sites did not exhibit any responsiveness to expression of BRCA1 and Oct-1 protein or to MMS treatment. Similarly, the PG-CAATwt with 3 repeats of the CAAT motifs demonstrated a clear induction following expression of either BRCA1 or NF-YA, which is one of the subunits of NF-Y transcription factor and binds to CAAT box. PG-CAAT

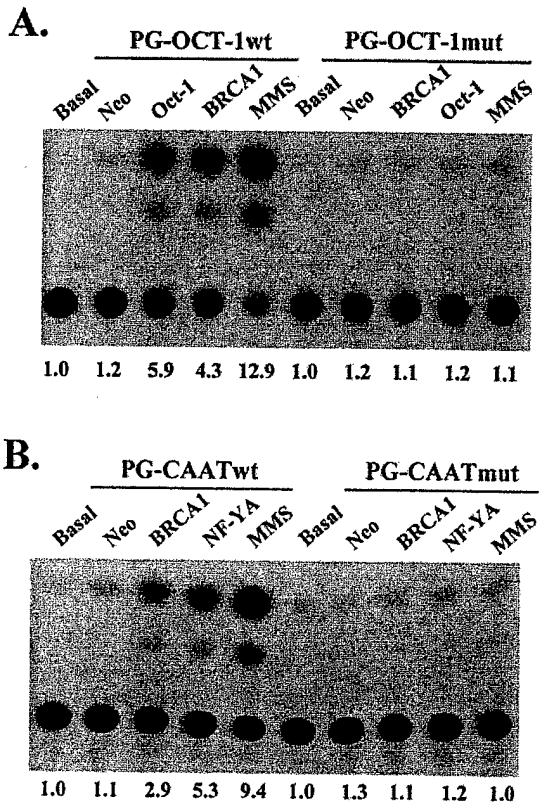

FIG. 3. **Both OCT-1 and CAAT box motifs confer inducibility of BRCA1 to a non-related minimal promoter.** *A*, 4 μg of PG-OCT-1wt and PG-OCT-1mut constructs containing 5 repeats of intact OCT-1 or mutant OCT-1 motifs upstream of the minimal polyomavirus promoter linked to a CAT gene were cotransfected with 4 μg of the indicated expression vectors (pCMV.neo, pCR3.BRCA1, and pCR3.Oct-1) into HCT116 cells. 40 h later, cells were collected for CAT assay as described in *A*. *B*, 4 μg of PG-CAATwt or PG-CAATmut plasmids, which are CAAT reporter constructs (see "Experimental Procedures"), were transfected with 4 μg of the indicated expression vectors (pCMV.neo, pCR3.BRCA1, and pCMV.NF-YA) into HCT116 cells. CAT assay was performed as in *A*.

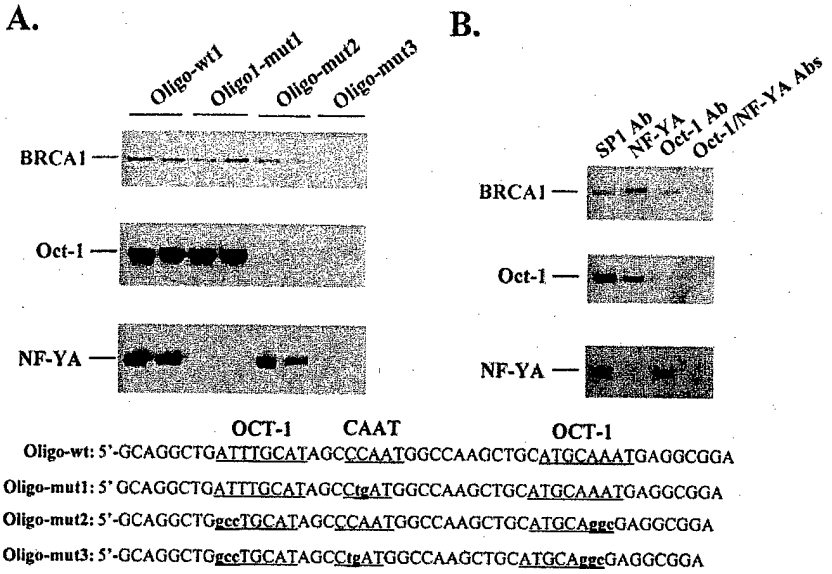

FIG. 4. **Pull-down assay with biotin-labeled oligonucleotides containing the OCT-1 and CAAT1 motifs.** *A*, nuclear extracts were prepared from HCT116 cells as described under "Experimental Procedures" and incubated with biotin-labeled 51-bp oligonucleotides, which contain either intact or mutated OCT-1 and CAAT sequences. Proteins bound to these nucleotides were isolated with streptavidin-agarose beads, and BRCA1, Oct-1, and NF-YA were detected by immunoblotting analysis (see "Experimental Procedures"). *B*, the nuclear extracts were immunodepleted with the antibodies against Jun-D, Oct-1, and NF-YA prior to incubation with the nucleotide containing intact OCT-1 and CAAT motifs (*Oligo-wt*).

also exhibited strong activation by MMS. However, PG-CAAT-mut with mutated CAAT motifs did not respond to expression of BRCA1 and NF-YA or MMS treatment. Collectively, the results presented above further indicate that the BRCA1 activation of the *GADD45* promoter is mediated through the OCT-1 and CAAT motifs.

BRCA1 Physically Interacts with OCT-1 and CAAT Motifs Via Its Physical Association with Both Oct-1 and NF-YA Proteins—Because the OCT-1 and CAAT motifs mediate the transcriptional activation of the *GADD45* promoter by BRCA1, effort was made to determine whether BRCA1 directly binds to the *GADD45* promoter region containing both OCT-1 and CAAT sites. An approach called "biotin-streptavidin pull-down assay" was employed to identify the proteins bound to the BRCA1-responsive region of the *GADD45* promoter. The biotin-labeled 51-bp double-stranded oligonucleotides corresponding to −107 to −57 of the *GADD45* promoter were incubated with nuclear extracts from HCT116 cells and pulled down by streptavidin (see "Experimental Procedures"). The protein complexes bound to the oligonucleotides were loaded onto SDS-PAGE gel and analyzed by immunoblotting assay with antibodies against BRCA1, Oct-1, and NF-YA. In Fig. 4*A*, the Oligo-wt that contains the intact OCT-1 and CAAT motifs was able to pull down the Oct-1, NF-YA, and BRCA1 proteins, indicating that all three proteins physically associate with this BRCA1-regulatory region. In Oligo-mut1, where the CAAT box was mutated, both the Oct-1 and BRCA1 proteins but not NF-YA were detected in the precipitated complexes. In the case of Oligo-mut2, where two OCT-1 sites were disrupted, BRCA1 and NF-YA proteins were present but not Oct-1. However, when all OCT-1 and CAAT motifs were mutated in the Oligo-mut3, no BRCA1, Oct-1, or NY-FA proteins were detected. These results strongly suggest the following two interpretations: (*a*) BRCA1 physically associates with the region of the *GADD45* promoter between −107 and −57 through its interaction with both OCT-1 and CAAT motifs; and (*b*) BRCA1 interacts with OCT-1 or CAAT motifs independently because single mutation of either motif did not disrupt BRCA1 interaction with the BRCA1-responsive region of the *GADD45* promoter.

However, because BRCA1 is not a sequence-specific binding transcription factor, it is most likely that the association of BRCA1 protein with the *GADD45* promoter is through its interaction with the Oct-1 and NF-Y factors, which directly bind to the *GADD45* promoter via their motifs. To address this

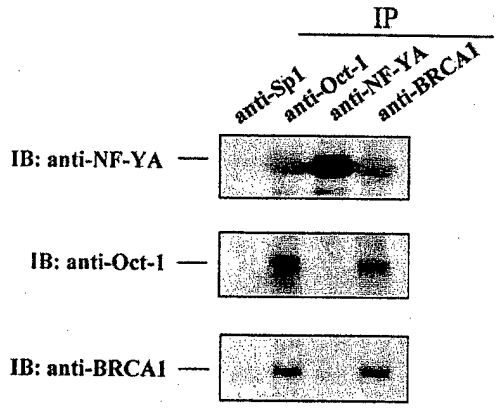

FIG. 5. **Physical association of BRCA with Oct-1 and NF-YA.** Nuclear protein from HCT116 cells was prepared (see "Experimental Procedures") and immunoprecipitated with anti-Jun-D, anti-Oct-1, anti-NF-YA, and anti-BRCA1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The immunocomplexes were analyzed by SDS-PAGE and immunoblotted with antibodies against NF-YA, Oct-1, and BRCA1, respectively. The visualized bands are shown; their estimated masses were 42–46 kDa for NF-YA, 97 kDa for Oct-1, and 220 kDa for BRCA1. *IP*, immunoprecipitation; *IB*, immunoblotting analysis.

issue, the Oligo-wt was incubated with the nuclear extracts, which were immunodepleted with anti-Oct-1 or -NF-YA antibodies prior to the pull-down assay. As shown in Fig. 4*B*, depletion with single antibody to either Oct-1 or NF-YA proteins did not affect binding of BRCA1 to the *GADD45* promoter region. However, immunodepletion of both the Oct-1 and NF-YA proteins completely abolished the association of BRCA1 with the *GADD45* promoter, indicating that association of BRCA1 with the *GADD45* promoter is through its interaction with the Oct-1 and NF-YA proteins, which directly bind to the *GADD45* promoter.

Next, we further determined the physical interactions of BRCA1 with Oct-1 and NF-YA proteins. Nuclear extracts isolated from HCT116 cells were incubated with anti-Jun-D, anti-Oct-1, anti-NF-YA, or anti-BRCA1 antibodies and immunoprecipitated with protein A/G-agarose beads. The immunocomplexes were then analyzed by Western blotting assay, and the results are shown in Fig. 5. NF-YA protein was present in the immunocomplexes precipitated by the antibodies against Oct-1, NF-YA, and BRCA1, suggesting physical interactions of NF-YA with Oct-1 and BRCA1. Oct-1 protein was detected in

the immunocomplexes with both anti-Oct-1 and anti-BRCA1 antibodies. Similarly, BRCA1 protein was detected in the anti-Oct-1 and anti-BRCA1 immunocomplexes. In contrast, no NF-YA, Oct-1, or BRCA1 proteins was present in the anti-Jun-D-immunoprecipitated complex. However, it is somewhat surprising that we did not detect Oct-1 and BRCA1 proteins in the anti-NF-YA-immunocomplex. One likely interpretation is that the interacting domains of Oct-1 and BRCA1 in NF-YA protein might share the region with the epitopes to the antibody against NF-YA, which possibly lead to dissociation of the NF-YA-BRCA1 and NF-YA-Oct-1 protein complexes. Taken together, these results indicate an association of BRCA1 with Oct-1 and NF-YA and an interaction between Oct-1 and NF-YA as well.

DISCUSSION

Studies presented in this paper and our earlier report (41) have demonstrated that BRCA1 activates the *GADD45* promoter. By using 5′-deletion analysis, the BRCA1-regulatory elements have been mapped at the *GADD45* promoter region between −107 and −62, where there are two OCT-1 motifs and one CAAT motif. Disruption of the OCT-1 and CAAT motifs abrogates the activation of the *GADD45* promoter by BRCA1 expression, indicating that both OCT-1 and CAAT sites are required for the BRCA1 activation of the *GADD45* promoter. This finding is further supported by the observation that the OCT-1 and CAAT motifs are able to confer BRCA1 inducibility to a non-related minimal polyomavirus promoter, when multiple repeats of these motifs are cloned upstream of the minimal promoter linked to a CAT gene. In the biotin-streptavidin pull-down assay, BRCA1 protein exhibits an association with the oligonucleotides corresponding to the *GADD45* promoter region from −107 to −57. Mutations of all OCT-1 and CAAT sites in such oligonucleotides disrupt association of BRCA1 with the *GADD45* promoter. Importantly, BRCA1 protein is demonstrated to interact physically with both Oct-1 and NF-YA proteins, and depletion of Oct-1 and NF-YA proteins results in abrogation of association of BRCA1 with the *GADD45* promoter. We conclude that BRCA1 transactivation of the *GADD45* promoter is mediated through BRCA1 interaction with Oct-1 and NF-YA proteins.

BRCA1 has been implicated in DNA damage-induced cellular response, including apoptosis, cell cycle arrest, and DNA repair (7–13). Inactivation of *BRCA1* correlates with genomic instability (14), indicating that one of the major roles for *BRCA1* is to maintain genomic fidelity. In addition to direct interactions of BRCA1 with proteins involved in cell cycle control and DNA repair, BRCA1-mediated transcriptional regulation may also greatly contribute to its role in cellular response to DNA damage. For example, both p21and *GADD45*, which are important players in the control of cell cycle checkpoints (29, 57), are regulated by BRCA1 (10, 41). It has been well accepted that the roles of BRCA1 as a tumor suppressor might be at least in part mediated through its transcriptional properties, given the evidence that tumor-derived mutations within the C terminus of BRCA1 are defective in transcriptional activation (10, 32). In agreement with this point, the tumor-derived BRCA1 mutants (p1749R and Y1853insA) that lack transcriptional activity are unable to activate the *GADD45* promoter (41). However, the regulation of *GADD45* by BRCA1 appears to be complex and might involve differential mechanism(s). This complex regulation may be due to the following points. (*a*) BRCA1 activation of *GADD45* has been shown to involve the BRCA1-responsive elements located at both the intronic or promoter regions of *GADD45* (30, 41, 58). (*b*) Most likely, BRCA1 regulates *GADD45* through its interaction with other transcription factors that directly bind to the *GADD45* promoter or intronic regions instead of direct binding of BRCA1 to the regulatory regions. (*c*) BRCA1 protein might be subject to phosphorylation in the process of DNA damage-induced transcriptional activation (5, 6). (*d*) BRCA1-mediated transactivation might recruit transcriptional coactivators, such as p300/CBP (38). Therefore, future work will further characterize the biochemical consequences of the interaction between BRCA1 and Oct-1 and NF-YA to determine whether Oct-1 and NF-YA are subject to protein stabilization, phosphorylation, or acetylation.

The *GADD45* promoter is strongly activated following genotoxic stress, including UV radiation, MMS, and medium starvation (54). Most recently, we have demonstrated that the p53-independent UV induction of the *GADD45* promoter is also regulated through both OCT-1 and CAAT motifs located at the same region between −107 and −62 of the *GADD45* promoter. Mutations of all OCT-1 and CAAT motifs abolish the induction of the *GADD45* promoter by UV radiation and MMS. In addition, protein levels of the Oct-1 and NF-YA transcription factors are elevated following DNA damage (53). Moreover, mitogen-activated protein kinases (c-Jun N-terminal kinase and extracellular signal-regulated kinase) also activate the *GADD45* promoter through the OCT-1 and CAAT motifs. In the current study, we demonstrate that the OCT-1 and CAAT motifs mediate the BRCA1 activation of the *GADD45* promoter. Therefore, it can be speculated that the OCT-1 and CAAT motifs are critical in the regulation of the p53-independent induction of *GADD45* in response to growth arrest signals (such as BRCA1 expression) and a variety of DNA-damaging agents. It is worth noting that in the OCT-1 and CAAT motifs appear to function in an additive but independent manner because single mutation of either OCT-1 sites or the CAAT box only reduced induction of the *GADD45* promoter by BRCA1, whereas mutations of all OCT-1 and CAAT motifs completely disrupted the BRCA1 activation of the *GADD45* promoter (Fig. 2).

The finding that BRCA1 regulates the *GADD45* through its interaction with transcription factors Oct-1 and NF-YA is of importance, given evidence that both the OCT-1 and CAAT motifs are widely present in the many gene promoters. Oct-1 and NF-YA are ubiquitous transcription factors involved in the development, cell cycle regulation, and cellular senescence (50, 51, 59, 60). Recently, we have found that OCT-1 and NF-YA proteins are induced after exposure of cells to multiple DNA-damaging agents and therapeutic agents in a p53-independent manner (52, 53). These observations indicate that both Oct-1 and NF-YA proteins are able to participate in cellular responses to genotoxic stress. In addition, our current study has shown a physical interaction of NF-YA with Oct-1 protein, suggesting that induction of *GADD45* by BRCA1 might involve a functional interaction between these two proteins. In fact, Oct-1 and NF-YA proteins have been reported previously to synergistically regulate histone H2B gene transcription during *Xenopus* early development (61). In summary, the study presented here has demonstrated the biochemical mechanism by which BRCA1 regulates the *GADD45* promoter and indicated that *GADD45* is a BRCA1 downstream effector. Furthermore, identification of the OCT-1 and CAAT1 as BRCA1-responsive elements has broadened the biological roles for *BRCA1* in transcriptional regulation.

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RE: MS#5436

Dear Dr. Zhan,

I am very pleased to inform you that your manuscript entitled "**GADD45-induced cell cycle G2-M arrest associates with altered subcellular distribution of cyclin B1 and is independent of p38 kinase activity**" is acceptable for publication in *Oncogene*, and has been forwarded to the publisher. You will be getting further information directly from the publisher, Nature Publishing Group.

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GADD45-induced cell cycle G2-M arrest associates with altered subcellular distribution of cyclin B1 and is independent of p38 kinase activity*

Running title: *GADD45* alters subcellular distribution of cyclin B1

Keywords: p53, *GADD45*, G2-M arrest, cyclin B1

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Abstract

In response to DNA damage, the cell cycle checkpoint is an important biological event in maintaining genomic fidelity. Gadd45, a p53-regulated and DNA damage inducible protein, has recently been demonstrated to play a role in the G2-M checkpoint in response to DNA damage. In the current study, we further investigated the biochemical mechanism(s) involved in the *GADD45*-activated cell cycle G2-M arrest. Using the tetracycline-controlled system (tet-off), we established *GADD45*-inducible lines in HCT116 (wild-type p53) and Hela (inactivated p53 status) cells. Following inducible expression of the Gadd45 protein, cell growth was strongly suppressed in both HCT116 and Hela cells. Interestingly, HCT116 cells revealed a significant G2-M arrest but Hela cells failed to arrest at the G2-M phases, indicating that the *GADD45*-activated G2-M arrest requires normal p53 function. The *GADD45*-induced G2-M arrest was observed independent of p38 kinase activity. Importantly, induction of Gadd45 protein resulted in a reduction of nuclear cyclin B1 protein, whose nuclear localization is critical for the completion of G2-M transition. The reduced nuclear cyclin B1 levels correlated with inhibition of Cdc2/cyclin B1 kinase activity. Additionally, overexpression of cyclin B1 substantially abrogated the *GADD45*-induced cell growth suppression. Therefore, *GADD45* inhibition of Cdc2 kinase activity through alteration of cyclin B1 subcellular localization may be an essential step in the *GADD45*-induced cell cycle G2-M arrest and growth suppression.

Introduction

In response to DNA damage, mammalian cells arrest at the transition from G1 to S phase (G1-S checkpoint) and G2 to M phase (G2-M checkpoint) (Hartwell & Weinert, 1989). Cell cycle arrest at these checkpoints presents DNA replication and mitosis in the presence of DNA damage. Inactivation of those cell cycle checkpoints results in genomic instability, which is closely associated with cell transformation and tumorigenesis. In addition, disruption of normal cell cycle controlling machinery often has dramatic consequences on therapeutic sensitivity (Elledge, 1996; Hartwell & Kastan, 1994; Kohn *et al.*, 1994; O'Connor & Kohn, 1992; Paulovich *et al.*, 1997).

Currently, the mechanism(s) by which DNA damaging agents activate cell cycle G1-S checkpoint is well understood. The tumor suppressor p53 gene plays a critical role in the control of G1-S arrest. Following DNA damage, p53 transcriptionally upregulates p21 (el-Deiry *et al.*, 1993), one of the p53-downstream genes and a potent cell cycle-dependent kinase inhibitor. Subsequently, induced p21 forms complexes with Cdk-cyclin and inhibits the activity of cdk4-cyclin D, Cdk6-cyclin D, Cdk2-cyclin E, and Cdk2-cyclin A, and in turn transiently arrest cells at the G1-S transition (Harper *et al.*, 1993; Sherr & Roberts, 1995; Xiong *et al.*, 1993; Zhang *et al.*, 1994). It has been demonstrated that the disruption of endogenous p21 abrogates the G1-S checkpoint after cell exposure to DNA damage (Waldman *et al.*, 1995). P53 has also been implicated in the control of the G2-M checkpoint. Introduction of p53 into p53-deficient human fibroblasts results in both G1-S and G2-M arrest (Agarwal *et al.*, 1995; Stewart *et al.*, 1995) and the HPV-16 E6 viral oncoprotein, which blocks p53 function, has been shown to decrease the stringency of the mitotic checkpoint (Thompson *et al.*, 1997). Recent evidence indicates

that p53 and p21 are required for maintaining the G2 checkpoint in human HCT116 cells (Bunz *et al.*, 1998). In addition, 14-3-3, which blocks Cdc25 activity and arrests cells at the G2-M transition, is demonstrated as one of the p53 downstream genes (Hermeking *et al.*, 1997). Most recently, *GADD45*, a p53-regulated and DNA damage-inducible gene, is found to play an important role in the G2-M checkpoint in response to certain types of DNA damaging agents (Jin *et al.*, 2000; Wang *et al.*, 1999; Zhan *et al.*, 1999).

However, the G2-M checkpoint is complex and may involve redundant controls including both p53-independent and p53-dependent mechanisms. It has been well accepted that many of the G2-M regulators appear to ultimately target Cdc2, a protein kinase required for the mitotic entry in mammalian cells (Elledge, 1996; O'Connor, 1997). Activation of Cdc2 kinase requires its association with mitotic cyclins (cyclin B1 and cyclin A), and phosphorylation at Thr-161 and dephosphorylation at Thr-14/Tyr-15 cells (Ducommun *et al.*, 1991; Elledge, 1996; O'Connor, 1997). After DNA damage, several G2-M regulators, including Chk1, Chk2, 14-3-3 and ATM, alter Cdc2 activity by inhibiting dephosphorylation of Cdc25C phosphatase. The inhibition of Cdc25C activity prevents the removal of inhibitory phosphorylations from Thr-14 and Tyr-15 of Cdc2 (Elledge, 1996; O'Connor & Fan, 1996; Paulovich *et al.*, 1997). In addition, DNA damage is able to suppress Cdc2 activity by inhibiting the accumulation of cyclin B1 mRNA and protein (Bernhard *et al.*, 1995; Muschel *et al.*, 1991; Muschel *et al.*, 1992). Delayed entry into mitosis following DNA damage also correlates with nuclear exclusion of cyclin B1 protein (Toyoshima *et al.*, 1998).

The *GADD45* gene is induced by a variety of DNA damaging agents, including ionizing radiation (IR), methyl methansulfonate (MMS), UV radiation (UV),

hydroxyurea and medium starvation (Fornace *et al.*, 1988; Fornace *et al.*, 1989; Papathanasiou *et al.*, 1991). The IR-induction of *GADD45* is transcriptionally regulated by p53 via a p53-binding site in the third intron (Kastan *et al.*, 1992; Zhan *et al.*, 1994a). In contrast, *GADD45* induction by UV radiation or MMS treatment is detected in all mammalian cells regardless of p53 status. However, recent evidence shows that p53 can still contribute to cellular responses to UV, MMS and medium starvation although it is not required (Zhan *et al.*, 1998; Zhan *et al.*, 1996). Gadd45 is a nuclear protein and binds to multiple important cellular proteins such as proliferating cell nuclear antigen (PCNA) (Hall *et al.*, 1995; Smith *et al.*, 1994), p21 protein (Chen *et al.*, 1996; Kearsey *et al.*, 1995; Zhao *et al.*, 2000), core histone protein (Carrier *et al.*, 1999), MTK/MEKK4 (Takekawa & Saito, 1998), an upstream activator of the JNK pathway, and Cdc2 protein kinase. The presence of Gadd45 in these complexes indicates that Gadd45 may be an important player in cell cycle control, DNA repair and the regulation of signaling pathway. The role of *GADD45* in maintaining genomic stability has been demonstrated by the recent finding that the mouse embryonic fibroblasts (MEF) derived from gadd45-null mice exhibit aneuploidy, chromosomal aberrations, gene amplification and centrosome amplification. Additionally, gadd45-knockout mice display increased radiation carcinogenesis (Hollander *et al.*, 1999). In this study, we have further investigated the role of *GADD45* in the G2-M checkpoint and demonstrated that the *GADD45*-induced G2-M arrest depends on normal cellular p53 function, but is independent of p38 kinase activity, which is reported to be required for the initiation of the G2-M checkpoint after UV radiation. In addition, inducible expression of Gadd45

protein has been shown to result in alterations of cyclin B1 subcellular distribution, which might be a consequence of the interaction of Gadd45 with Cdc2 proteins.

Results

Inducible expression of Gadd45 protein suppresses human cell growth

To further investigate the biological mechanism(s) by which *GADD45* plays a role in the control of cell cycle regulation, we established tetracycline-regulated *GADD45*-inducible cell lines in human cervical cancer Hela cells (see Materials and Methods), where cellular p53 function is inactivated, and human colorectal carcinoma HCT116 cells, which has wild-type p53 and normal p53 function. As shown in Figure 1A, both Hela *GADD45*-inducible cells and HCT116 *GADD45*-inducible cells exhibited extremely low basal levels of the endogenous Gadd45 protein. Following withdrawal of tetracycline, Gadd45 protein was greatly induced and presented more than tenfold induction in both cell lines. Next, the effect of Gadd45 protein on growth suppression was examined in these two *GADD45*-inducible cell lines. To perform this experiment, 500, 1000 or 2000 cells were seeded and grown in DMEM medium at 100-cm dishes 16 h prior to tetracycline withdrawal. After removing tetracycline, cells continued to grow for 14 days and then were fixed, scored for colonies containing more than 50 cells. Similar to our previous finding that overexpression of *GADD45* protein via transient transfection inhibits tumor cell growth (Zhan *et al.*, 1994b), inducible expression of Gadd45 protein in both Hela and HCT116 lines strongly suppressed colony formation (Figure. 1B), indicating a

suppressive role of *GADD45* in cell grown regardless of p53 status. In agreement with this observation, both Hela and HCT116 lines with inducible expression of Gadd45 protein exhibited a substantial slow growth rate (results not shown). Taken together, these results indicate that *GADD45* plays a negative role in the control of cell progression.

GADD45 induced cell cycle G2-M arrest depends on normal cellular p53 function

In order to further determine the role of *GADD45* in the control of cell cycle G2-M arrest, cell cycle distribution analyses were conducted in both HCT116 and Hela *GADD45*-inducible cell lines. Following removal of tetracycline, *GADD45*-inducible cells were collected at 24 h or 36 h and subject to flow cytometry analysis. The results presented in Fig. 2 depict a representative profile of cell cycle distribution in those cells. In the HCT116 *GADD45*-inducible line (wt p53 status), cells grown in the presence of tetracycline presented 14-16% population in G2-M phase. However, inducible expression of Gadd45 protein resulted in a clear accumulation of the G2-M fraction. Approximately 32% of the cells were arrested at the G2-M phase of the cell cycle in the absence of tetracycline, indicating that *GADD45* expression alone is able to halt cells in G2-M phase. In contrast, after inducible expression of Gadd45, Hela cells (inactivated p53 status) did not exhibit any evident changes of cell cycle distribution. In consistence with these results, introduction of *GADD45* expression vector into HCT116 via transient transfection resulted in increased G2-M population in HCT116 but not in HCT116p53-/-, where p53 alleles were knocked out by homologous recombination approach (result not

shown). These observations further demonstrate that *GADD45*-mediated G2-M arrest requires normal cellular p53 function.

GADD45 induced cell cycle G2-M arrest is not affected by inhibitors of p38

The mitogen-activated protein kinase p38 has recently been reported to play a critical role in cell cycle G2-M checkpoint in response to UV radiation (Bulavin *et al.*, 2001). To understand whether p38 kinase activation contributes to the *GADD45*-induced G2-M arrest, the mitotic index was measured in the *GADD45* inducible cells in the presence of p38 kinase inhibitor, SB202190. In Fig. 3A, high mitotic indices were observed in HCT116 cells treated with nocodazole. In response to UV radiation, mitotic indices substantially decreased, indicating that UV treatment arrests cells in the G-M transition. Addition of p38 inhibitor SB202190 at a concentration of 10 μM was shown to greatly attenuate the UV-induced G2-M arrest. In Fig.3B, inducible expression of Gadd45 protein exhibited low mitotic indices, which reflects a significant G2-M arrest by Gadd45. However, p38 inhibitor SB202190 (10 μM) showed little effect on the Gadd45-induced G2-M arrest. These results suggest that the *GADD45* induction of cell cycle G2-M checkpoint does not require activation of p38 kinase.

Expression of Gadd45 protein alters the level of nuclear cyclin B1 but does not affect phosphorylation statuses of Cdc25C or Chk1

In our previous report, we have demonstrated that Gadd45 protein physically interacts with Cdc2 kinase, dissociates Cdc2/cyclin B1 complexes and in turn inhibits Cdc2 kinase activity, but does not alter Cdc2 phosphorylation status (Zhan *et al.*, 1999). However, the biochemical consequence of the interaction between Gadd45 and Cdc2 remains to be further defined. Since nuclear localization of cyclin B1 protein is thought to be critical for the completion of G2-M transition, we further examined cyclin B1 protein distributions in both the nucleus and cytoplasm. As shown in Fig. 4A, following inducible expression of Gadd45 protein in HCT116 cells, there were no evident alterations of Cdc2 protein in both the cytosol and nuclear compartments. Interestingly, nuclear cyclin B1 protein exhibited a significant reduction in response to induction of Gadd45. In support of this observation, cytosol cyclin B1 appeared to increase after Gadd45 induction. These results indicate that Gadd45 induction caused subcellular redistribution of cyclin B1 protein. In the same experiment, Chk1 and Cdc25C phosphorylation statuses were also examined, but no phosphorylations of Chk2 or Cdc25C were detected following Gadd45 induction. However, cells treated with UV and MMS displayed increased phosphorylations for Chk2 ad Cdc25C. Additionally, we analyzed Cdc2 and Cdk2 kinase activity following Gadd45 protein expression and found Cdc2 was inhibited by Gadd45 but Cdk2 kinase activity remained at the similar levels after Gadd45 expression (Fig. 4B). Taken together, Gadd45 protein is able to alter cyclin B1 nuclear localization and in turn inhibits Cdc2 kinase activity.

Cyclin B1 abrogates the GADD45-induced cell growth suppression

We have previously demonstrated that *GADD45*-induced growth suppression (Zhan *et al.*, 1994b), in a great content, correlates with its inhibition of Cdc2/cyclin B1 kinase activity. It is assumed that interaction of Gadd45 with Cdc2 causes dissociation of the Cdc2/cyclin B1 complex, and in turn alters subcellular localization of cyclin B1, which contributes to the loss of Cdc2 kinase activity. Therefore, we examined whether introduction of cyclin B1 into cells can rescue *GADD45*-induced growth-suppression. To do this, *GADD45* expression vector was co-transfected with expression vectors for cyclin B1, Cdc2, or cyclin D1 into HCT116 cells (p53 wt line). In Fig. 5, expression of *GADD45* in HCT116 cells suppressed 80% cell growth. While in the presence of cyclin B1 overexpression, *GADD45* only generated 30% growth suppression, indicating that cyclin B1 is able to abrogate *GADD45*-induced growth inhibition. In contract, overexpression of both Cdc2 and cyclin D1 failed to rescue the *GADD45*-induced growth suppression. Additionally, cyclin B1 had little effect on p21, suggesting that p21-induced cell growth might not be mainly through its role in the G2-M arrest. Interestingly, *GADD45* was able to suppress cell growth in Hela cells, which contains inactivated p53 and does not exhibit *GADD45*-induced G2-M arrest. However, both cyclin B1 and cyclin D1 were incapable of abrogating *GADD45*-induced cell growth, indicating *GADD45* inhibits cell growth in cells with abnormal p53 probably through a different mechanism distinct from the inhibition of Cdc2 kinase activity in p53 wt cell lines.

Discussion

In this report, we further investigated the role of Gadd45, a p53-regulated and stress-inducible protein, in the control of cell cycle G2-M checkpoint. Using tetracycline-controlling system (tet-off), we established *GADD45*-inducible lines in both HCT116 (wt p53) and Hela (negative p53 status) cells. Therefore, induction of Gadd45 protein was nicely manipulated by the withdrawal of tetracycline. Following inducible expression of Gadd45, cell growth was strongly inhibited in both HCT116 and Hela lines. In consistent with our previous finding that introduction of *GADD45* expression vector into human normal fibroblast via microinjection approach causes cells to arrest at the G2-M transition, induction of Gadd45 protein in the HCT116 *GADD45*-inducible line greatly increased cell population in G2-M phase, but Gadd45 expression was unable to induce G2-M arrest in Hela cells, which contain inactivated p53. The *GADD45*-induced G2-M arrest appeared independent of p38 kinase activity, as employment of p38 kinase inhibitor (SB202190) did not abrogate GADD-induced G2-M arrest. More importantly, overexpression of Gadd45 protein was shown to result in reduction of nuclear cyclin B1 protein and inhibited Cdc2 kinase activity, but had no effect on Chk1, Cdc25C phosphorylation and Cdk2 activity. In addition, co-introduction of cyclin B1 expression vector was able to substantially disrupt the *GADD45*-induced growth suppression.

The tumor suppressor p53 gene has been implicated in the control of cell cycle checkpoint in response to genotoxic stress (Bunz *et al.*, 1998; Bunz *et al.*, 1999; Kastan *et al.*, 1991; Kastan *et al.*, 1992). The role for p53 in G1-S arrest is clearly shown to be mediated though p21 (Harper *et al.*, 1993; Sherr & Roberts, 1995; Xiong *et al.*, 1993;

Zhang *et al.*, 1994). However, the role of p53 in the control of the G2-M arrest is under debate and remains to be further elucidated. It is postulated that as one of the p53-targeted genes (Kastan *et al.*, 1992; Zhan *et al.*, 1994a), *GADD45* might be a strong player in mediating p53-regulated cell cycle G2-M checkpoint. Previous studies have shown that Gadd45 protein interacts with Cdc2 and dissociates the Cdc2/cyclin B1 complex (Jin *et al.*, 2000; Zhan *et al.*, 1999). Subsequently, "free" cyclin B1 protein dissociated from the Cdc2 complex is more likely pumped out from the nucleus, probably by the nuclear transport system. As a result of exclusion of cyclin B1 protein from nucleus, Cdc2 kinase activity is inhibited and followed up by the cell cycle G2-M arrest. This goes along with the finding by Toyoshima et al that DNA damage causes increased nuclear export of cyclin B1 and in turn arrests cells at the G2-M transition (Toyoshima *et al.*, 1998). Our observations that inducible expression of *GADD45* protein alters cyclin B1 nuclear localization (Fig. 4) have suggested that exclusion of nuclear cyclin B1 protein by Gadd45 might be an essential step for the *GADD45*-induced G2-M arrest. Therefore, the findings in this work have further presented the precise evidence that the p53-*GADD45* pathway is well involved in the control of G2-M arrest.

The mechanism(s) for p53 dependence of the *GADD45*-induced cell cycle G2-M arrest is not clear at the present time. Bunz et al has reported that cells with disrupted p53 displays an impaired G2-M checkpoint after DNA damage and suggested that the role for p53 in sustaining G2-M arrest after DNA damage might be mediated through p21 (Bunz *et al.*, 1998). However, our previous investigations have already demonstrated that p21 is not required for *GADD45*-induced G2-M arrest, since introduction of *GADD45* expression vector into p21 deficient cells, where endogenous p21 has been disrupted, is

able to generate G2-M arrest (Wang *et al.*, 1999). We have also not found any alterations of MDM2 protein level following Gadd45 induction and no physical interactions between Gadd45 and MDM2 proteins (result not shown). Therefore, both p21 and MDM2 appear not to be the candidates to mediate the role for p53 in *GADD45*-induced G2-M arrest. Future investigation is required to explore the mechanism by which p53 is required for the *GADD45*-induced G2-M arrest.

The mitogen-activated kinase p38 is required for initiating the G2-M checkpoint after UV radiation, probably through phosphorylating Cdc25B at serines 309 and 361 (Bulavin *et al.*, 2001). However, the *GADD45*-induced G2-M arrest is independent of p38 kinase activity. These results have further confirmed that Gadd45 acts at the late G2-M transition or early mitotic phase, instead of at the initiation of G2-M transition. In addition, inhibitory effect of the Gadd45 protein appears to be specifically localized on Cdc2/cyclin B1 complex, as induction of Gadd45 protein does not alter phosphorylations of Chk1 and Cdc25C. Overexpression of cyclin B1 protein has been found in certain types of human tumors although the biological function of this overexpressed protein in tumorigenesis remains unclear (Soria *et al.*, 2000). Interestingly, Overexpression of cyclin B1 is closely associated with loss of a p53 function (Yu et al, in press). In Fig. 5, co-expression of cyclin B1 with Gadd45 protein abrogated the Gadd45-induced cell growth suppression. This evidence has provided a new insight into understanding on the role of cyclin B1 in development of genomic instability and tumorigenesis.

GADD45 was shown to suppress cell growth in both HCT116 (wt p53) and Hela (inactivated p53) cells, regardless of p53 status (Fig. 5). However, *GADD45* only generated G2-M arrest in HCT116 cells, but not in Hela cells, suggesting that the

GADD45-induced growth suppression is complex and might involve the biological events distinct from the G2-M arrest. In fact, Takekawa and Saito have previously reported that *GADD45* interacts with MTK1/MEKK4, an upstream activator of the JNK pathways, and induced apoptosis in Hela cells (Takekawa & Saito, 1998). Therefore, *GADD45* is able to play a negative role in cell growth probably through both cell cycle arrest and apoptosis. The importance of *GADD45* in maintenance of genomic fidelity has been presented by the evidence that gadd45-null mice generated by gene targeting exhibit aneuploidy, chromosome aberrations, gene amplification and centrosome amplification, and increased tumorigenesis after DNA damaging agents (Hollander *et al.*, 1999). Therefore, the current studies have further demonstrated the mechanism(s) by which *GADD45* plays a role in maintaining genomic stability and provides insight into understanding the p53-*GADD45* pathway in cellular response to genotoxic stress.

Materials and Methods

Establishment of the GADD45 inducible cell line and cell culture

To establish *GADD45* tet-off inducible cell lines, human colorectal carcinoma HCT116 cells were initially transfected with pTet-Off plasmid (CLONTECH, Palo Alto, CA), which is commercially available and expresses the tTA regulator proteins, and the G418-resistant colonies were selected and amplified. Next, the cells expressing tTA proteins were subject to second round transfection with pTRE-*GADD45* construct, where the *GADD45* gene was inserted into BamH1/HindIII sites of pTRE plasmid (CLONTECH, Palo Alto, CA). The cells transfected with pTRE-*GADD45* plasmid were selected by hygromycin at 200 ug/ml for 14 days, and each hygro-resistant colony was separately

collected for detection of Gadd45 protein expression under tet-off system. In the case of Hela *GADD45* inducible lines, Hela cells expressing tTA were commercially obtained from CLONTECH and transfected with pTRE-*GADD45* construct and the hygro-resistant cells were selected as described in HCT116 cells. *GADD45*-inducible cells were grown in DMEM medium supplemented with 10% fetal bovine in the presence of tetracycline at a concentration of 2 μg/ml. To induce expression of Gadd45 protein, DMEM containing tetracycline was removed and the plates were washed 4 times with PBS, and fresh DMEM medium containing no tetracycline was then added to cells. Cells were collected at the indicated time points for examination of induced Gadd45 protein.

Antibodies and Immunoblotting Analysis

The following antibodies were used in the experiments; *GADD45*, Cdc2 (Santa Cruz biotechnology, Santa Cruz, CA, USA), cyclin B1 (Pharmingen, San Diego, CA) and Cdc25C and Chk1 phosphorylation-sites specific antibodies (Cell Signaling Technology Inc, Beverly, MA). *GADD45*-inducible cells were exponentially grown in DMEM medium containing tetracycline at a concentration of 2 μg/ml. After withdrawal of tetracycline, cells were collected at the indicated time. For preparation of cellular protein, plates were rinsed with PBS and cells were lysed in PBS containing100 μg/ml phenyl-methylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 1% NP-40 (lysis buffer). Lysates were collected by scraping and cleared by centrifugation at 4 ^{0}C. 100 μg of cellular protein was loaded onto 12% SDS-PAGE gel and transferred to Protran membranes. Membranes were blocked for 1 hour at room temperature in 5% milk, washed with PBST (PBS with 0.1% Tween-20), and incubated with indicated antibodies for 2 hours. Membranes were washed 4 times in PBST and HRP-conjugated anti-mouse

antibody was added at 1:4000 in 5% milk. After one hour, membranes were washed and detected by ECL (Amersham, Arlington Height, IL) and exposed to X-ray film (Kodak, Rochester, NY).

Growth Suppression Assay

500, 1000, or 2000 cells from Hela or HCT116 *GADD45*-inducble lines were seeded in 100-cm dishes and grown in DMEM medium containing 2 μg/ml tetracycline for 16 hours. Following withdrawal of tetracycline, cells were fixed at 14 days and scored for colonies containing at least 50 cells (Zhan *et al.*, 1994b).

Cdc2 and Cdk2 Kinase Assays-

Cellular lysates isolated from the *GADD45*-inducible cells were incubated with 10 μl of cyclin B1 antibody (Pharmingen, San Diego, CA) or 20 μl of Cdk2 antibody (Santa Cruz biotechnology, Santa Cruz, CA, USA), and 20 μl of protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 ^{0}C for 6 hours. Immuno-complexes were washed 4 times with lysis buffer and followed by kinase buffer. Histone H1 kinase assays were then performed in the presence of 10 μg of histone H1 (Upstate Biotechnology, Lake Placid, NY), 15 mM $MgCl_2$, 7 mM β-glycerol phosphate, 1.5 mM EDTA, 0.25 mM sodium orthvanadate, 0.25 mM DTT and 10 μCi of γ-^{32}P ATP in 30 μl volume. After 15 min at 30 ^{0}C, the reactions were mixed with an equal amount of standard 2 X SDS protein denature loading buffer, sized-separated on a 12% SDS-PAGE gel (Zhan *et al.*, 1999).

Flow Cytometry Analysis

HCT116 and Hela *GADD45*-inducible cells were plated into 100-mm dishes at a density of 6 X 10^5 and grown in DMEM containing 2 μg/ml of tetracycline. 16 h later, medium was removed and plates were washed 4 times followed by addition of fresh medium. After incubation for 36 h, cells were collected, washed with PBS, fixed with 70% ethanol for 2 hr at 4^0C. Cells were then incubated with RNase (10 μg/ml) for 30 min and stained with propidium iodine (Sigma; 50 μg/ml). Cell cycle analysis was performed using Becton Dickson fluorescence-activated cell analyzer. The At least 10,000 FITC positive cells were analyzed using CellQuest and Modfit programs (Wang *et al.*, 1999).

Analysis of mitotic index in HCT116 GADD45-inducible cells

HCT116 *GADD45*-inducible cells were seeded at a density of 6 X 10^5 in DMEM containing 2 μg/ml of tetracycline. Following withdrawal of tetracycline, cells were grown in the presence of 10 μM p38 kinase inhibitor SB203580 and harvested at the indicated time points, fixed in methanol:acetic acid (3:1), spread on glass microscope slides, air-dried and stained with 5%Giemsa. Nuclei exhibiting condensed, evenly staining chromosomes were scored as mitotic. At least 1000 cells were counted in each determination. Meanwhile, HCT116 cells treated with p38 kinase inhibitor SB203580 were exposed to UV radiation and subjected to analysis of mitotic index.

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Legends

Figure. 1. Tumor cell growth suppression by Gadd45 protein. *A*, Induction of Gadd45 protein in HCT116 and Hela cells controlled by the Tet-off system. HCT116 *GADD45*-inducible lines and Hela *GADD45*-inducible cell lines were established as described in Materials and Methods. Cells were placed in 100 mm dishes at a density of 4 X 10^5 and grown in DMEM medium containing tetracycline at a concentration of 2 μg/ml. After withdrawal of tetracycline, cells were collected at the indicated time points for preparation of cellular protein. 100 μg of whole cell protein was used for immunoblotting analysis with anti-*GADD45* antibody. As a loading control, anti actin antibody was included. *B*. Induction of Gadd45 protein suppresses cell growth. HCT116 and Hela *GADD45*-inducible cells were seeded at a density of 1000 cells per 100 mm dish and grown in medium containing 2 μg/ml of tetracycline. 16 hours later, medium was

removed and plates were washed three times with PBS, then fresh medium containing no tetracycline was added into plates. The cells were fixed and stained at 14 days and scored for colonies containing at least 50 cells. The experiments were performed 4 time and only representative results were shown here.

Figure. 2. Cell cycle G2-M arrest following inducible expression of Gadd45 protein in both HCT116 and Hela cells. HCT116 and Hela *GADD45*-inducible cells were growing in DMEM medium with 10% fetal bovine serum in the presence of tetracycline at a concentration of 2 μg/ml. After withdrawal of tetracycline, cells were collected at the indicated time points and subject to flow cytometric analysis as described in Materials and Methods.

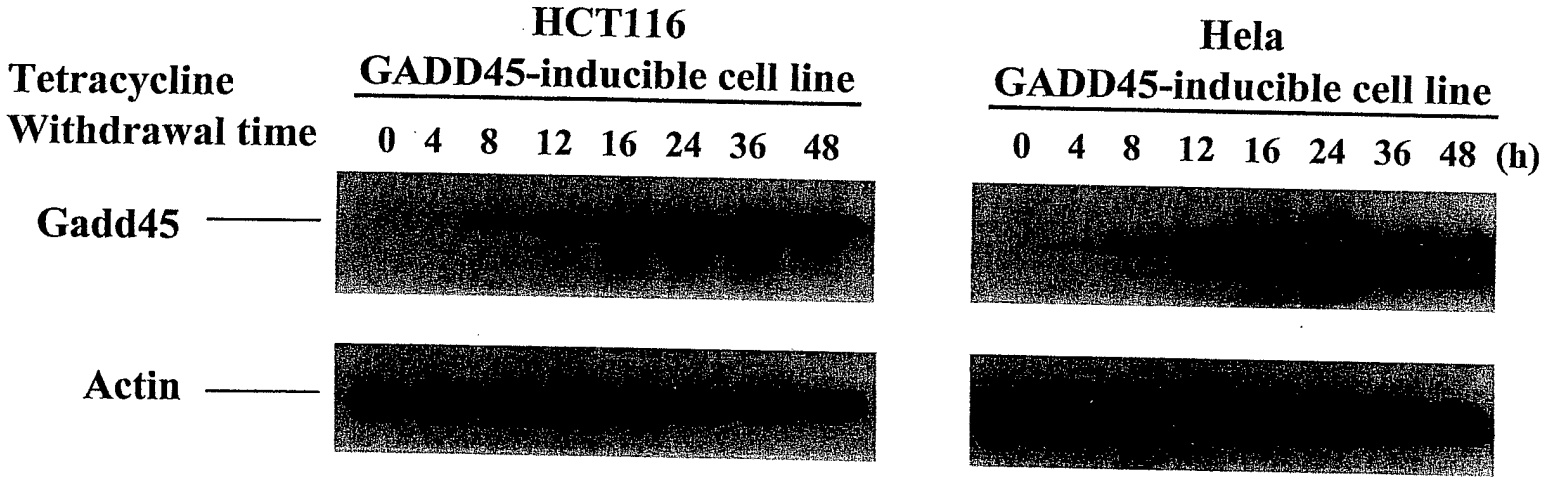
Figure. 3. Mitotic entry after UV radiation or inducible expression of Gadd45 protein. *A*, HCT116 cells were UV irradiated in the presence of 10 μM p38 kinase inhibitor SB203580 and mitotic indices were determined as described in Materials and Methods. *B*, HCT116 *GADD45*-inducible cells were grown in medium with tetracycline (2 μg/ml). Upon the withdrawal of tetracycline, cells were exposed to 10 μM p38 kinase inhibitor SB203580 and followed by determination of mitotic indices at the indicated time points.

Figure. 4. Subcellular localization of cyclin B1 protein and inhibition of Cdc2 kinase activity following inducible expression of Gadd45. *A*, Cellular proteins were prepared from HCT116 *GADD45*-inducible cells after withdrawal of tetracycline at the indicated time points. 100 μg of proteins were loaded onto SDS PAGE gel for detection of

subcellular distribution of cyclin B1 protein and phosphorylations of CHK1 or Cdc25C. *B*, One mg of cellular proteins isolated from HCT116 *GADD45*-inducible cells at the indicated time points was immunoprecipitated with anti-cyclin B1 or cyclin E antibodies, and histone H1 kinase assays were performed as described in Materials and Methods. Labeled histone H1 was detected by autoradiography following size separation on a SDS-PAGE gel.

Figure. 5. Effect of cyclin B1 expression on the *GADD45*-induced cell growth suppression. Human colorectal carcinoma HCT116 cells were transfected with the indicated expression vectors. Following selection with G418 for two weeks, cells were fixed and the colonies that contained at least 50 cells were counted. Quantitative results represent the average of three individual experiments.

A.


HCT116
GADD45-inducible cell line
Hela
GADD45-inducible cell line
Tetracycline
Withdrawal time
0 4 8 12 16 24 36 48
0 4 8 12 16 24 36 48 (h)
Gadd45
Actin

B.

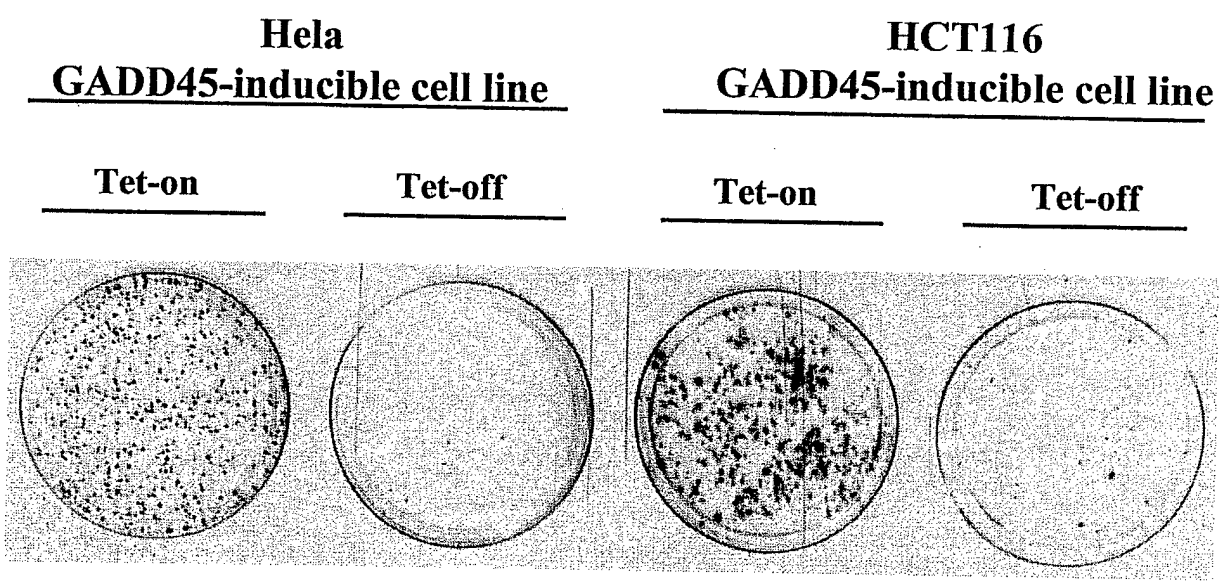

Hela
GADD45-inducible cell line
HCT116
GADD45-inducible cell line
Tet-on
Tet-off
Tet-on
Tet-off
100%
8.7%
100%
11.2%

Fig.1

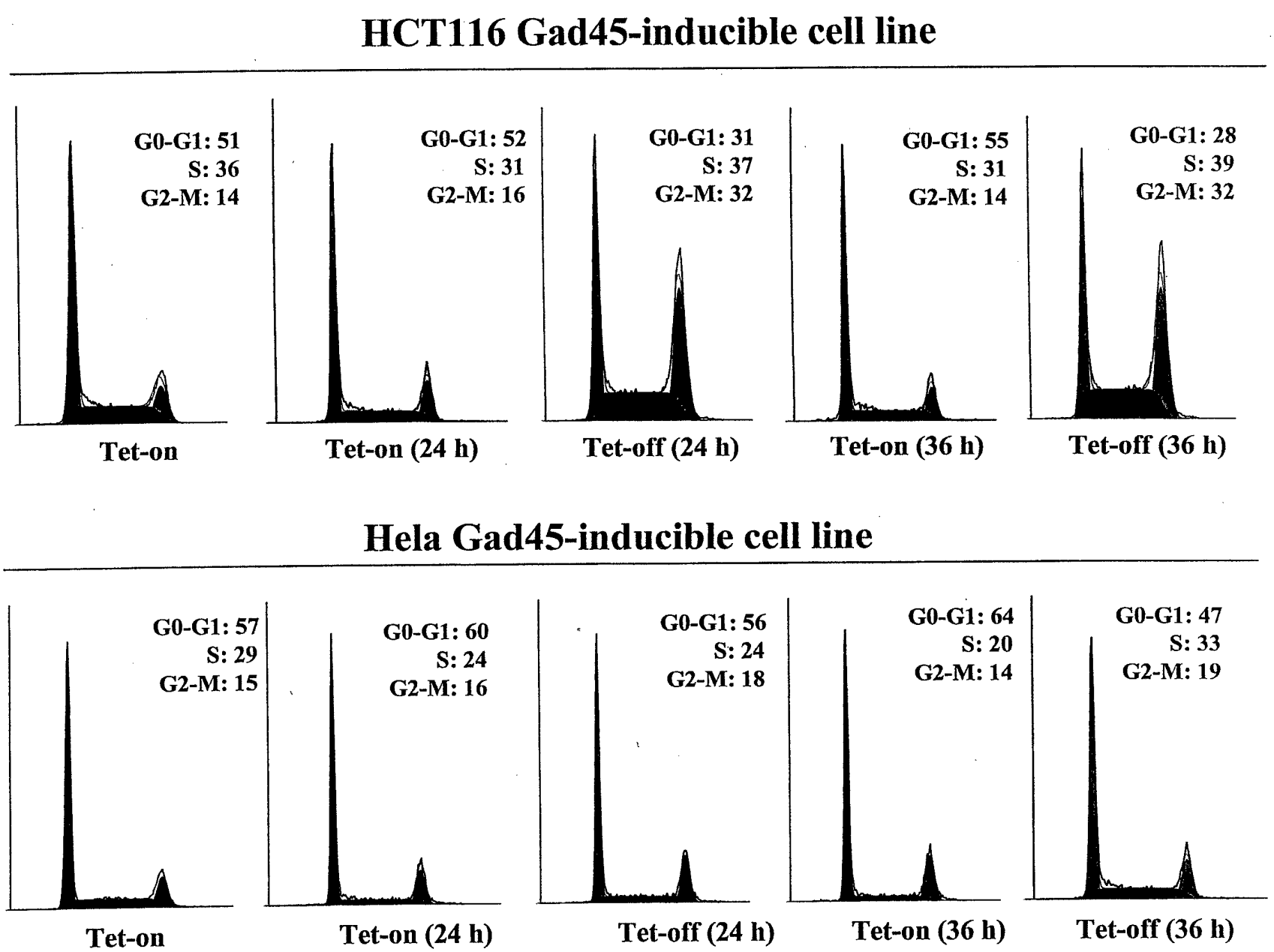

HCT116 Gad45-inducible cell line
G0-G1: 51
S: 36
G2-M: 14
Tet-on
G0-G1: 52
S: 31
G2-M: 16
Tet-on (24 h)
G0-G1: 31
S: 37
G2-M: 32
Tet-off (24 h)
G0-G1: 55
S: 31
G2-M: 14
Tet-on (36 h)
G0-G1: 28
S: 39
G2-M: 32
Tet-off (36 h)
Hela Gad45-inducible cell line
G0-G1: 57
S: 29
G2-M: 15
Tet-on
G0-G1: 60
S: 24
G2-M: 16
Tet-on (24 h)
G0-G1: 56
S: 24
G2-M: 18
Tet-off (24 h)
G0-G1: 64
S: 20
G2-M: 14
Tet-on (36 h)
G0-G1: 47
S: 33
G2-M: 19
Tet-off (36 h)

Figure 2

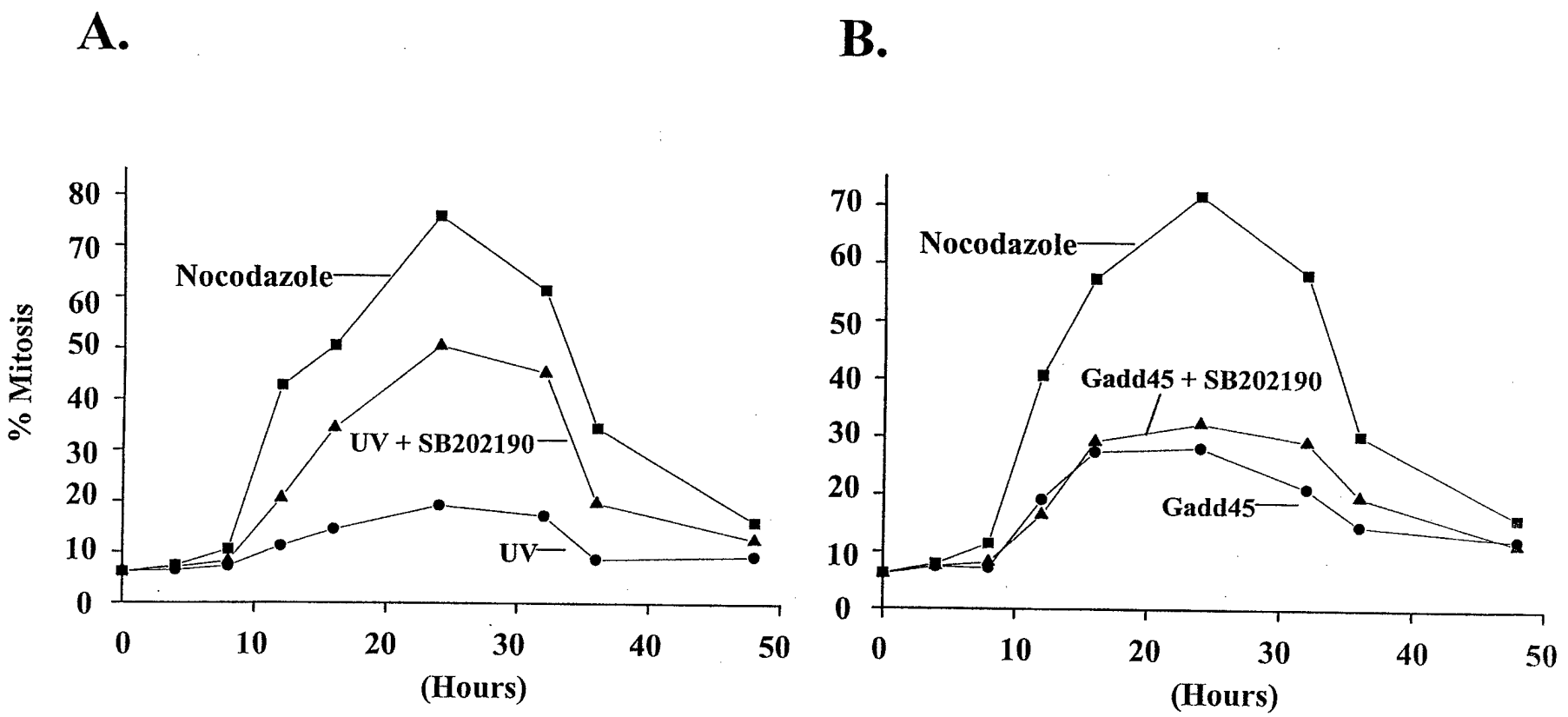

A.
B.
% Mitosis
Nocodazole
UV + SB202190
UV
(Hours)
Nocodazole
Gadd45 + SB202190
Gadd45
(Hours)

Figure. 3

A.

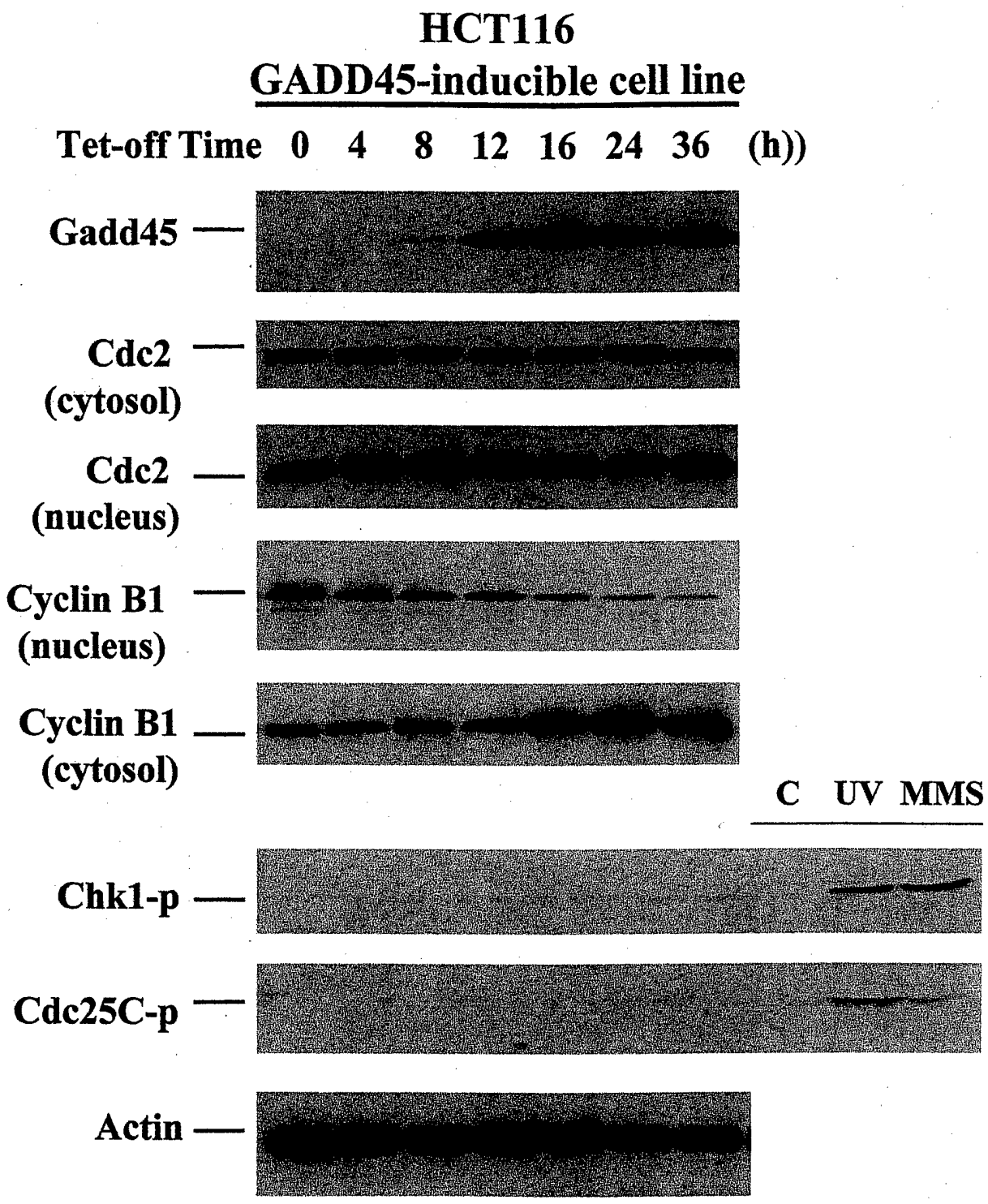

HCT116
GADD45-inducible cell line
Tet-off Time 0 4 8 12 16 24 36 (h))
Gadd45
Cdc2 (cytosol)
Cdc2 (nucleus)
Cyclin B1 (nucleus)
Cyclin B1 (cytosol)
C UV MMS
Chk1-p
Cdc25C-p
Actin

Figure. 4A

B.

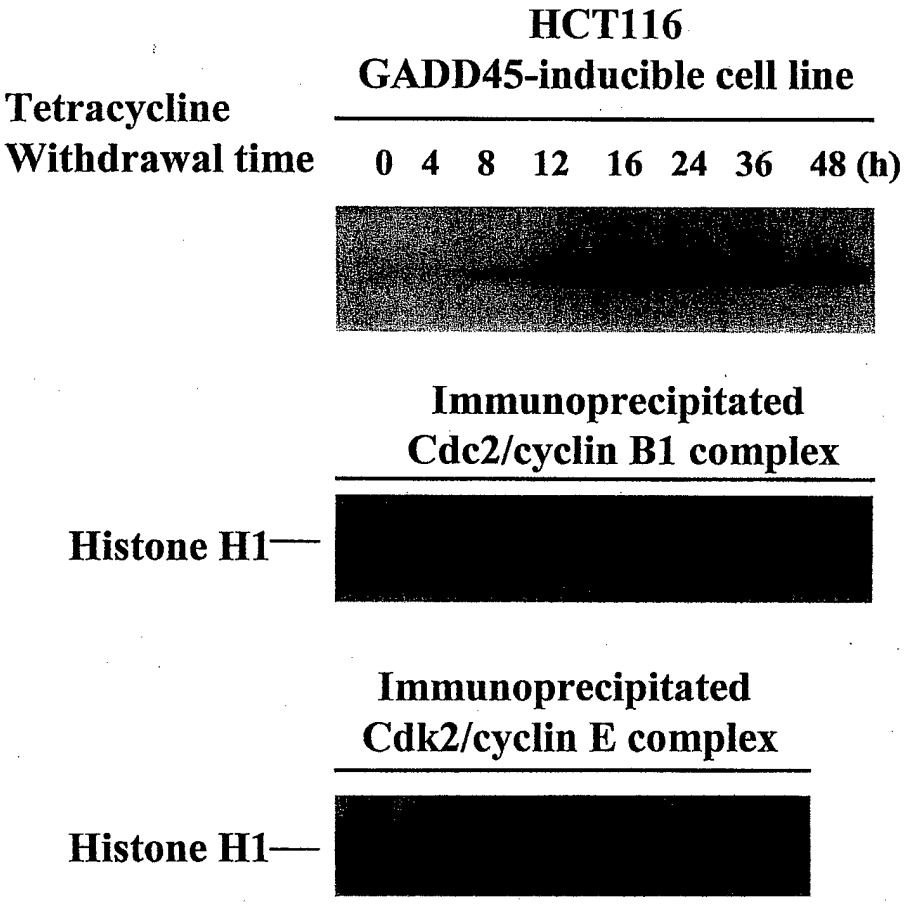

HCT116
GADD45-inducible cell line
Tetracycline
Withdrawal time
0 4 8 12 16 24 36 48 (h)
Immunoprecipitated
Cdc2/cyclin B1 complex
Histone H1—
Immunoprecipitated
Cdk2/cyclin E complex
Histone H1—

Figure. 4B

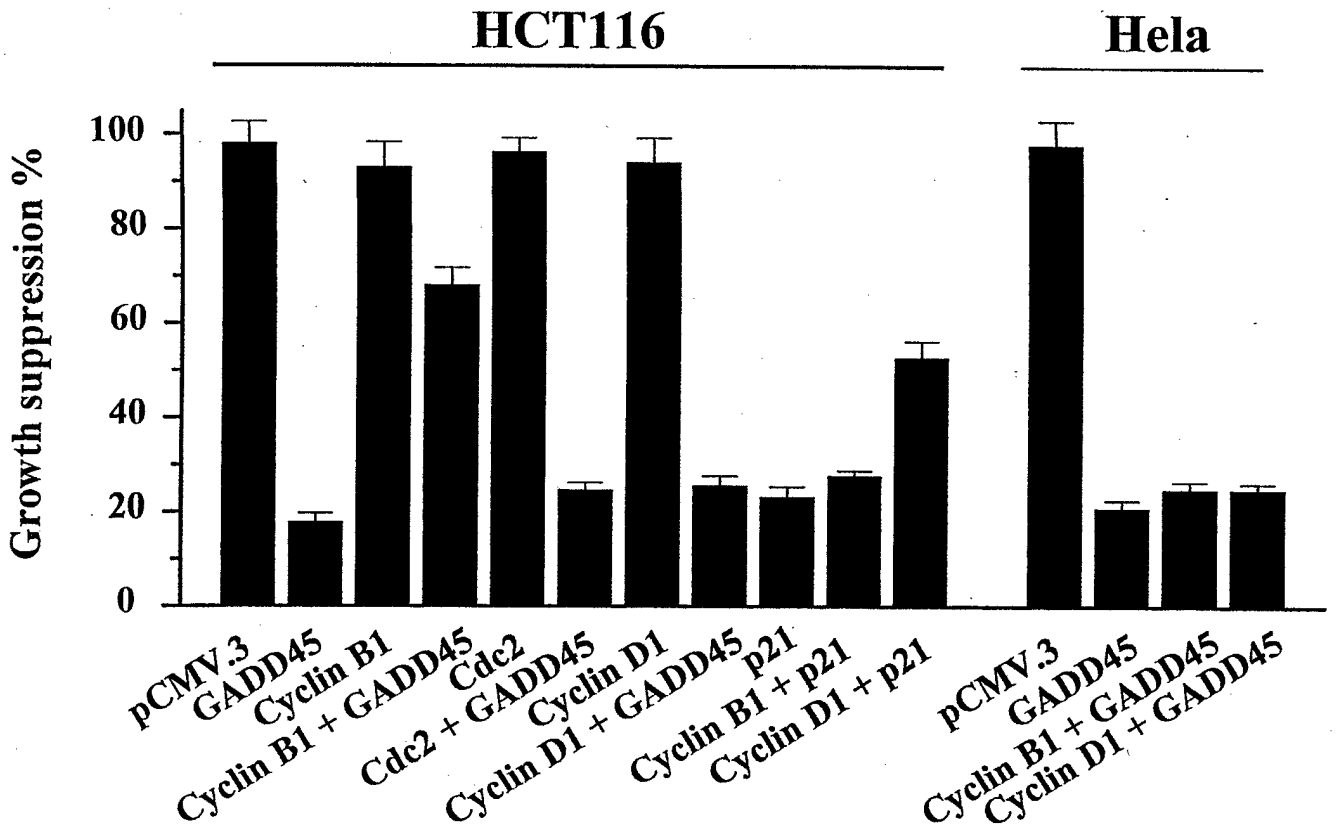

HCT116
Hela
Growth suppression %
100
80
60
40
20
0
pCMV 3
GADD45
Cyclin B1
Cyclin B1 + GADD45
Cdc2
Cdc2 + GADD45
Cyclin D1
Cyclin D1 + GADD45
p21
Cyclin B1 + p21
Cyclin D1 + p21
pCMV 3
GADD45
Cyclin B1 + GADD45
Cyclin D1 + GADD45

Figure. 5

Abstract for the 93th Annual Meeting of American Association of Cancer Research

BRCA1 regulates *GADD45* through its interactions with the OCT-1 and CAAT motifs. Wenhong Fan, Shunqian Jin, Tong Tong, Hongcheng Zhao, Feiyue Fan, Patricia Blanck, Isaac Alamo, Baskaran Rajasekaran and Qimin Zhan. Department of Radiation Oncology, Cancer Institute and Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213

The implication of BRCA1 in gene regulation greatly accounts for its role as a tumor suppressor. The BRCA1-targeted genes are thought to mediate BRCA1's biological function. Previous studies by others and ours demonstrate that BRCA1 induces *GADD45*, a p53-regulated and stress-inducible gene that plays an important role in cellular response to DNA damage. However, the molecular mechanism(s) by which BRCA1 regulates *GADD45* remains to be elucidated. In this report, we have shown that BRCA1 strongly activates the *GADD45* promoter in a p53-independent manner and the BRCA1 activation of the *GADD45* promoter is mediated through the OCT-1 and CAAT motifs located at the *GADD45* promoter region between –107 and –57. Site-directed mutations of both OCT-1 and CAAT motifs abrogate induction of the *GADD45* promoter by BRCA1. When several repeats of OCT-1 or CAAT are cloned upstream of a minimal polyomavirus promoter linked to a chloramphenicol acetyltransferase (CAT) reporter gene, both OCT-1 and CAAT motifs confer BRCA1 inducibility on the non-related minimal promoter. Physical associations of BRCA1 protein with transcription factors Oct-1 and NF-YA, which directly bind to the OCT-1 and CAAT motifs, are established by biotin-streptavidin pull-down and coimmunoprecipitation assays. Such protein interactions are required for interaction of BRCA1 with the *GADD45* promoter since either immunodepletion of Oct-1 and NF-YA proteins or mutations in the OCT1and CAAT motifs disrupt BRCA1 binding to the *GADD45* promoter. These findings indicate that BRCA1 can upregulate its targeted genes through protein-protein interactions and provide a novel mechanism by which BRCA1 participates in transcriptional regulation.

********** NOTICE OF GRANT AWARD
****

RESEARCH Issue Date:02/28/2002
Department of Health and Human Services
National Institutes Of Health

NATIONAL CANCER INSTITUTE
****
****** ****

Grant Number: 1 R01 CA93640-01
Principal Investigator: ZHAN, QIMIN MD
Project Title: The BRCA1-GADD45 Pathway and Genomic Stability

DIRECTOR, OFC OF RESEARCH
UNIV OF PITTSBURGH
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UNITED STATES
Award e-mailed to: ornih@orserver.off-res.pitt.edu

Budget Period: 03/01/2002 - 02/28/2003
Project Period: 03/01/2002 - 02/28/2006

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The National Institutes of Health hereby awards a grant in the amount of $248,932(see ''Award Calculation'' in Section I) to UNIVERSITY OF PITTSBURGH AT PITTSBURGH in support of the above referenced project. This award is pursuant to the authority of 42 USC 241 42 CFR 52 and is subject to terms and conditions referenced below.

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Award recipients are responsible for reporting inventions derived or reduced to practice in the performance of work under this grant. Rights to inventions vest with the grantee organization provided certain requirements are met and there is acknowledgement of NIH support. In addition, recipients must ensure that patent and license activities are consistent with their responsibility to make unique research resources developed under this award available to the scientific community, in accordance with NIH policy. For additional information, please visit http://www.iedison.gov.

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